ENCE LIBRARY

AUG 18 1921

BIOLOGICAL BULLETIN

OF THE

Marine Biological Laboratory

WOODS HOLE, MASS.

VOL. XL JUNE, 1921 No. 6

CONTENTS

		PAGE
THOMAS, L. J.	*Morphology and Orientation of the Otocysts of Gonionemus*	287
BRUES, CHARLES T. AND GLASER, RUDOLF W.	*A Symbiotic Fungus Occurring in the Fat-Body of Pulvinaria innumerabilis Rath*	299
RICHARDS, A., AND THOMPSON, JAMES T.	*The Migration of the Primary Sex-Cells of Fundulus heteroclitus* .	325
HONDA, H.	*Spermatogenesis of Aphids; The Fate of the Smaller Secondary Spermatocyte*	349

PUBLISHED MONTHLY BY THE
MARINE BIOLOGICAL LABORATORY

PRINTED AND ISSUED BY
THE NEW ERA PRINTING COMPANY, INC.
LANCASTER, PA.

AGENT FOR GREAT BRITAIN
WILLIAM WESLEY & SON
28 Essex Street, Strand, London, W. C.

Single Numbers, 75 Cents. Per Volume (6 numbers), $3.00

Entered October 10, 1902, at Lancaster, Pa., as second-class matter under Act of Congress of July 16, 1894.

Editorial Staff

E. G. Conklin—*Princeton University.*

George T. Moore—*The Missouri Botanic Garden.*

T. H. Morgan—*Columbia University.*

W. M. Wheeler—*Harvard University.*

E. B. Wilson—*Columbia University.*

Managing Editor

Frank R. Lillie—*The University of Chicago.*

All communications and manuscripts should be sent to the Managing Editor, the University of Chicago, Sept. 15th to June 15th, or Woods Hole, Mass., June 15th to Sept. 15th. Subscriptions and other matter should be addressed to the Biological Bulletin, 41 North Queen Street, Lancaster, Pa

Vol. XL. *June, 1921.* *No. 6*

BIOLOGICAL BULLETIN

MORPHOLOGY AND ORIENTATION OF THE OTOCYSTS OF GONIONEMUS.[1]

L. J. THOMAS.

Introduction.

Gonionemus is used as an example of the *Hydro-medusæ* in many zoölogical laboratories of this country. In spite of this fact relatively little is known regarding the morphology and orientation of the sense organs of the representatives of this genus. Both the location and the structure of the otocysts have been very imperfectly treated in the literature. Frequently morphological and experimental treatises mentioning the otocysts refer the reader to the works upon other genera, the otocysts of which are presumed to be essentially similar to those of *Gonionemus*. In studying specimens of *Gonionemus vertens* Ag. and of *G. murbachii* Mayer the writer was impressed by the lack of agreement between his direct observations and the published statements by various early workers. These discrepancies led the writer to investigate the problem further in an attempt to discover the source of the statements which have been so generally incorporated into the literature and to determine, if possible, the precise structure and relationships of the otocysts in this genus.

On the Pacific coast of this country *G. vertens* A. Ag. occurs in the Puget Sound region and on the Atlantic coast *G. murbachii* Mayer is found in abundance in the Eel Pond at Woods Hole, Mass. Many references in the older literature incorrectly refer to the Atlantic species as *G. vertens* because at that time the Atlantic form was not considered as specifically distinct from the

[1] Contributions from the Zoölogical Laboratory of the University of Illinois, No. 181.

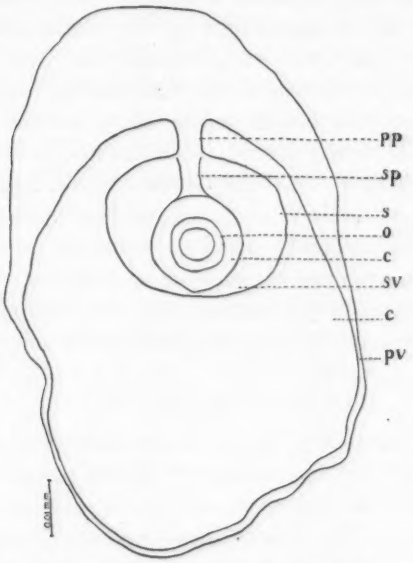
type of the genus. In 1895 Murbach mentioned certain differences between the two forms and in consideration for his work upon the Atlantic species A. G. Mayer (1901) described it as a distinct species under the name of *Gonionemus murbachii*.

The terms otocyst, statocyst, and lithocyst have been used interchangeably throughout the literature on Cœlenterates. In this paper the term otocyst is used in referring to the entire structure which is supposed to function as an organ of equilibrium. A careful study of the otocysts in *Gonionemus* has revealed the fact that the terminology ordinarily employed in describing the otocyst is entirely inadequate for an intelligible description of the parts and understanding of their functional relations. In *Gonionemus* the otocyst is not a simple vesicle enclosing an otolith as has usually been considered the case. From a morphological point of view there is considerable evidence that in *Gonionemus* the large vesicular structure is merely an adaptation for the protection of the true sensory apparatus all of which is lodged within the structure that has ordinarily been termed the otolith. This obvious confusion of terms renders a detailed description of the organ necessary.

Details of the organization of the otocyst are shown in Text Figure 1. The wall of the primary vesicle (*pv*) is the outermost wall of the entire organ and encloses a fluid-filled cavity (*c*) within which the spheroid (*s*) is suspended by the primary pedicel (*pp*). The thick wall of the spheroid encloses the fluid-filled cavity which is designated as the secondary vesicle (*sv*). Within the secondary vesicle rests the otolith (*o*), free to move about within the fluid-filled chamber. Extending from the distal end of the primary pedicel to the membranous lining of the secondary vesicle is a distinctly differentiated region to which the term secondary pedicel (*sp*) has been applied.

This investigation was carried on under the general direction of Dr. H. J. Van Cleave, to whom the writer is greatly indebted for suggestions and for securing the material and the identification of the species under consideration. Individuals of *Gonionemus murbachii* were obtained through the Supply Department of the Woods Hole Marine Biological Station for comparison

with those of *G. vertens* upon which the greater part of the work was done. Specimens of *G. vertens* collected at Friday Harbor, Washington, were submitted to Dr. Alfred G. Mayer and to Dr.



TEXT FIGURE 1. General organization of the otocyst of *Gonionemus*: *p v*, primary vesicle; *c*, fluid filled cavity; *s*, spheroid; *p p*, primary pedicel; *s v*, secondary vesicle; *o*, otolith; *s p*, secondary pedicel.

Henry B. Bigelow, both of whom very kindly verified the tentative identification of the species.

While the present paper deals primarily with the finer structure of the otocyst the latter part is devoted to a discussion of the orientation of the otocyst in the organism.

METHODS OF STUDY.

Specimens preserved in formalin were too opaque for accurate observations of the otocysts. Serial sections and toto-mounts of the bell margin, including ring canal, otocysts, and tentacles were prepared for the detailed study of the morphology and the orientation of the otocysts. The toto-mounts were made by

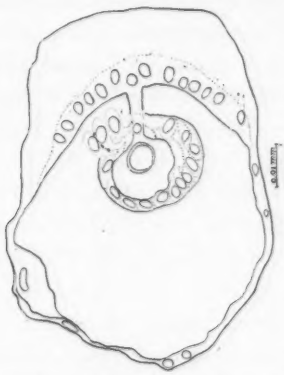
trimming the tentacles close to the bell margin with fine scissors and then clipping this rim, with the velum included, free from the remainder of the bell. Such preparations were stained with borax carmine and mounted in damar. The otocysts, though embedded in the mesoglea, were by this method sharply defined and readily visible for accurate observations. In some specimens prepared in this manner the finer details of structure could be determined. By careful manipulation, mounts prepared in this manner showed practically no distortion. Individuals 9 to 15 mm. in diameter were dehydrated, cleared, and mounted in damar with a shrinkage of 1 mm. or less in diameter. This shrinkage was obviously sustained uniformly by the various tissues for no evidences of wrinkling or distortion of parts were observable in the finished preparations. All drawings were made with the camera lucida from prepared mounts and sections.

Structure of the Otocyst.

Murbach (1903: 205) dismisses the anatomy of the otocysts in *Gonionemus* with the footnote: "The finer anatomy is naturally omitted. The description of the nervous system and the otocyst given in 'Das Nervensystem und die Sinnesorgane der Medusen,' O. u. R. Hertwig, text, pp. 48–69, Plates 4 and 5, fits very nearly those of *Gonionemus*." A careful examination of the Hertwig descriptions and drawings of otocysts in other genera reveals many points where a closer "fit" might be desired if the descriptions and drawings are to cover the conditions found in *Gonionemus*.

Superficial examination under a compound microscope reveals the otocysts of *Gonionemus* as tiny bubbles, each of which usually encloses a single spherical object. This last mentioned body has usually been considered as the otolith, though it probably comprises the entire sensory mechanism of the organ as indicated in the introduction to this paper. Detailed structure of this body is shown in Fig. 2, Plate I., which was drawn from a 15 μ section. This figure shows only the spheroid pendant from the wall of the primary vesicle by the minute primary pedicel (*pp*). The heavily nucleated cells which comprise the wall of the secondary

vesicle (*sv*) completely envelope the secondary pedicel (*sp*). The true otolith (*o*) is shown within the secondary vesicle. In the section from which this drawing was made a small tangential slice such as is shown in Fig. 3, Plate I., had been cut from the surface of the spheriod thus disclosing the cavity of the secondary vesicle. No sensory hairs or ridges were demonstrated. Perkins (1902: 786) also calls attention to the absence of such structures, though his observations were probably confined to the walls of the primary vesicle. In sections a thin tangential slice from the surface of the spheroid, Text Figure 2, may at



TEXT FIGURE 2. Tangential slice only partly removed from wall of spheroid.

first glance remind one of the projections figured by Hertwig (1879: 183, Figs. 1 and 2) for *Carmarina*.

The primary pedicel measures 0.043 mm. in diameter and 0.069 mm. in length. At its proximal end a single large nucleus is found. This pedicel is very delicate for in sectioning, it, together with the spheroid, is often torn loose from the cyst wall. In other cases only the spheroid is torn away from the distal end of the pedicel and may be found elsewhere on the slide. Goto (1903: 8) records encountering the same difficulty in the preparation of sections.

The secondary pedicel measures 0.115 mm. in length. Its diameter near the middle is about 0.023 mm. but the distal end is

expanded to about 0.043 mm. Two nuclei of apparently fixed relationships are found in the secondary pedicel, one near the proximal and the other near the distal end.

The cavity of the secondary vesicle is normally spherical in form though it is capable of some distortion. The diameter is about 0.138 mm. The contents of this cavity take stains so lightly and so evenly that there is strong evidence that only a fluid is present. Two or three concentric rings are observable in the spherical otolith contained within the secondary vesicle (Figs. 4–5, Plate I.). There is considerable variability in the size of the otolith. Some of the largest measured 0.069 mm. in diameter. The fluid in which the specimens were preserved was distinctly acid, consequently any calcareous deposits that might have been present in the otolith had been destroyed, leaving only the supporting structures. Perkins (1902: 786) refers to the otolith as "a calcium salt deposit in an organic matrix." In the specimens examined the otolith had no fixed position within the secondary vesicle but was apparently free to move about in the fluid filled space. The entire sensory mechanism as ordinarily described for an otocyst is thus contained within the confines of the spheroid. In the light of this morphological evidence it might be easily possible that the destruction of the primary vesicle need not appreciably impair the functioning of the organ.

Murbach (1903: 206) in experiments upon the function of the otocysts of *Gonionemus* collapsed the "otocysts" by thrusting them with a fine needle. In all probability the injury inflicted did not extend beyond the collapsing of the primary vesicle. According to his statements the specimens continued to act normally after this treatment. Upon the results of these experiments and upon the behavior of a single individual from which the otocysts were excised he based his conclusion that the otocysts play no important part in establishing the equilibrium in *Gonionemus*. The more or less normal behavior of the much mutilated individual from which the otocysts were cut is not readily explainable. On the other hand, in view of the fact that morphologically the entire sensory mechanism of the otocyst seems to be confined to the secondary vesicle there is little reason to expect serious

interference with the functioning of that organ when only the protective primary vesicle is collapsed.

My observations upon specimens of *G. vertens* and of *G. murbachii* have failed to disclose any essential difference in the details of structure or in the general orientation of the otocysts of these two species.

Relations of Otocysts to Bell Margin.

Because of the reliance placed upon the works of previous investigators and writers on the subject, Mayer's "Medusæ of the World" includes several erroneous statements and incorrect figures of the otocysts of *Gonionemus*. Few authors, with the exception of Mayer, have attempted to describe definitely the location of the otocysts with reference to their position on the margin of the bell. It seems probable that his description is based chiefly on Perkins' publication (1903: 786) which has been extensively quoted by Mayer though his observations upon the otocysts are very misleading. In Plate 34, Fig. 19, Perkins has reproduced a drawing by Professor Brooks which shows the otocysts as external projections from the margin of the bell between the bases of the tentacles. Further, the drawing of the "radial transverse section" of the bell (Fig. 25 of his same plate) confirms the impression of their external location. His explanation of their origin is as follows:

> In the case of the sensory clubs, the endodermal tissue of the circular canal grows down in a plug into the ectodermal tissue of the bell margin. This latter becomes closely applied to the outside of the plug, as a thin investing epithelium, and it also spreads out in a thin lamella over the inner surface of the capsule which appears in the ectoderm of the developing club.

In his summary Perkins (1903: 789) says, "sense organs appear at determinate points on the bell margin." The work in this article on sense organs seems to be principally that of Professor W. K. Brooks whose observations were apparently accepted by Perkins without attempt at verification.

Mayer (1910: 341) in his synopsis of the genus seems to have incorporated the foregoing incorrect observations bodily for he refers to the otocysts as "lithocysts external." On page 342 of the same work he again states that there are "numerous ex-

ternal lithocysts upon the bell margin between the tentacles." In characterizing the genus *Cubaia* (page 351), he states that there are "lithocysts projecting outward as in *Gonionemus*". . . "this genus is closely related to *Vallentinia* Brown 1902 but in *Vallentinia* the lithocysts are enclosed and on the inner side of the margin, whereas in *Cubaia* they are external and on the lower side of the margin between the tentacles." The plates of the same monograph are just as confusing as the foregoing descriptions in the manner in which the otocysts are located. Otocysts are shown as distinct projections from the external margin of the bell in Fig. 1 of Plate 45, though Fig. 2 of the same plate and Fig. 3 of Plate 46 correctly portray them embedded in the mesoglea as outgrowths from the ring canal.

The location of the otocyst within the mesoglea and the orientation with reference to other structures is shown in Fig. 1, Plate I.

Relations of Otocysts to Tentacles.

Hargitt (1910: 249) referring to the location of the otocysts, says: "Normally they should occur in somewhat symmetrical order between the bases of the tentacles. This, however, is rarely the case." Mayer (1910: 342) gives the number of tentacles for specimens of *G. vertens* 15 mm. in diameter as 60 to 70 but it appears that his count is too low. In the following table are given the data regarding the numbers of otocysts and tentacles in part of the material studied in the present investigation.

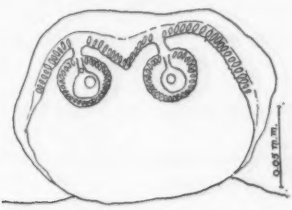
Table I.

Relative Numbers of Otocysts and Tentacles in Gonionemus.

Specimen Number.	Diameter of Bell After Clearing.	No. of Tentacles.	No. of Otocysts.
G. vertens			
1	14.5 mm.	91	76
2	14	86	73
3	14	99	72
4	12	84	60
5	11	94	75
6	11	101	78
7	10	94	89
8	9	79	61
9	9	83	57
10	8	79	60
G. murbachii			
1	9	48	61
2	10	64	70

With reference to the tentacles these organs display no absolutely fixed relationship. Though they usually alternate with the tentacles they may occur in pairs between two adjacent tentacles or two or more tentacles in continuous sequence may have no intervening otocyst.

In *G. vertens,* nos. 6 and 7 had three paired otocysts between adjacent tentacles; no. 7 also had two abnormal otocysts with two



TEXT FIGURE 3. Abnormal otocyst with two sensory spheroids within the same primary vesicle.

sensory spheroids in each capsule Text Figure 3. Hargitt (1901: 249) has noted this last mentioned variation and has called attention to its relative infrequence. Specimen no. 1 of *G. murbachii* had thirteen paired otocysts and no. 2 of the same species had eight.

SUMMARY.

1. There are two distinct vesicles in the otocyst of *Gonionemus.*

2. It seems probable that the conspicuous primary vesicle has chiefly a protective function, enclosing the essential sensory mechanism.

3. The secondary vesicle contains the otolith within a fluid-filled cavity.

4. No sensory hairs or ridges have been demonstrated.

5. Puncture of the primary vesicle as practiced by Murbach and others probably does not impair the essential sensory mechanism.

6. The otocysts of *G. vertens* and of *G. murbachii* are in close proximity to the periphery of the ring canal, their capsules imbedded in the mesoglea so that no portion of them protrudes beyond the margin of the bell.

7. The number and arrangement of otocysts and tentacles in *Gonionemus* is variable.

REFERENCES.

Agassiz, A.

'62 Contributions to the Natural History of the United States. Vol. 4, Boston.

'65 North American Acalephæ, Vol. 2, Cambridge.

Bigelow, H. B.

'09 The Medusæ. Mem. Mus. Comp. Zoöl. Harvard College, Vol. 37.

Brooks, W. K.

'86 Life-History of the Hydromedusæ. Mem. Boston Soc. Nat. Hist., Vol. 3: 359–430.

'95 The Sensory Clubs or Cordyli of Laodice. Jour. Morpnol., Vol. 10: 287–304.

Claus, C.

'78 *Ueber Charybdea Marsupialis.* Arb. Zoöl. Inst. Wien, Vol. 1: 221–276.

Goto, S.

'03 The Craspedote Medusa Olindias and Some of its Natural Allies. Mark Anniversary Volume: 3–25.

Haeckel, E.

'81 Monographie der Medusen, Vol. 2. Jena.

Hargitt, C. W.

'00 Variation Among Hydromedusæ. BIOL. BULL., Vol. 2: 221–255.

Hertwig, O. u. R.

'78 Das Nervensystem und die Sinnesorgane der Medusen. Leipzig.

Mayer, A. G.

'09 Medusæ of the World, Vol. 2, The Hydromedusæ. Carnegie Inst. Washington, Publication No. 109.

Murbach, L.

'03 The Static Function in *Gonionemus.* Amer. Jour. Physiol., Vol. 10: 201–209.

Murbach, L., and Shearer, C.

'03 On Medusæ from the Coast of British Columbia and Alaska. Proc. Zoöl. Soc. London, Vol. 2: 164–189.

Nutting, C. C.

'99 The Hydroids of the Woods Hole Region. Bull. U. S. Fish Com., Vol. 19: 325–386.

Perkins, H. F.

'03 The Development of *Gonionemus murbachii.* Proc. Acad. Nat. Sci. Philadelphia, Vol. 54: 750–790.

EXPLANATION OF PLATE.

Symbols.

c, cavity of secondary vesicle,
e c, ectoderm,
e n, endoderm,
e x, exumbrella,
m, mesoglea,
n, nerve ring,
n c, nettling cells,
o, otolith,
p p, primary pedicel,
r, ring canal,
s, spheroid,
s l, supporting layer,
s p, secondary pedicel,
s u, subumbrella,
s v, secondary vesicle,
v, velum.

Plate I.

The otocyst of *Gonionemus vertens*. All drawings were made with the camera lucida. The projected scale indicating magnification in each instance has the value of 0.01 mm.

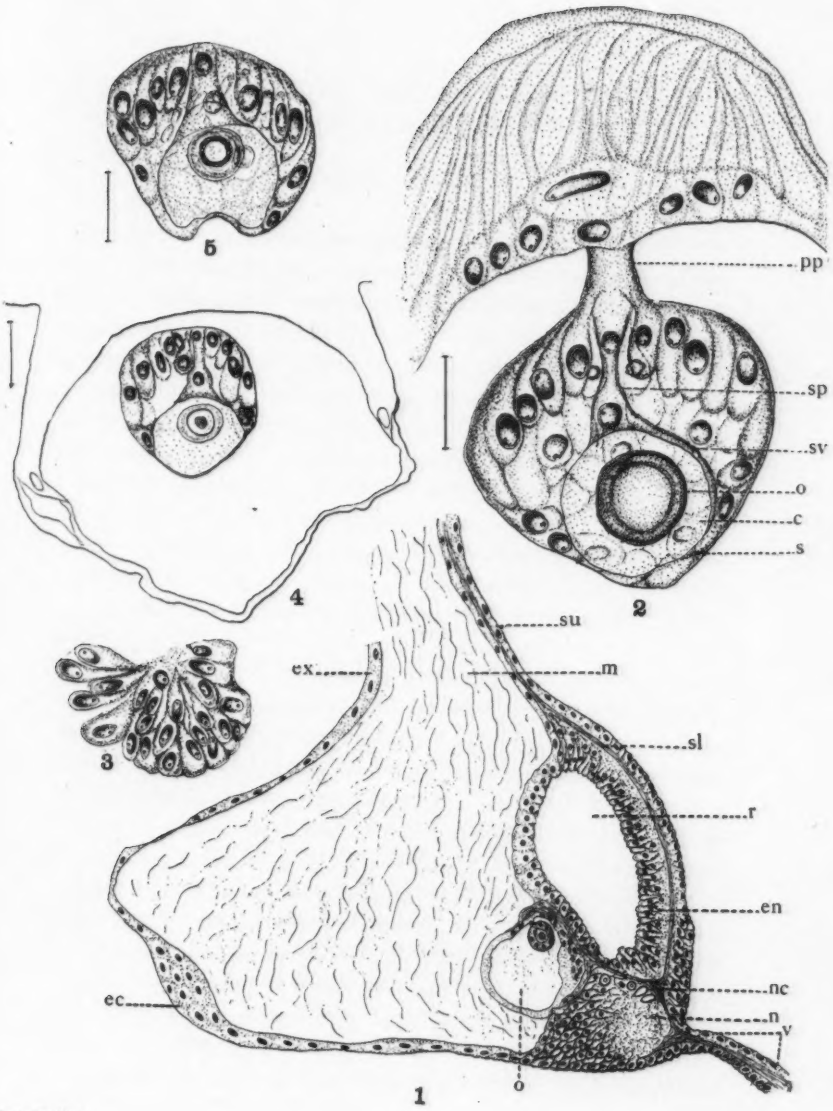
Fig. 1. Section through bell margin showing general location of the otocyst within the mesoglea and its orientation with reference to ring canal.

Fig. 2. Longitudinal section through the spheroid of an otocyst showing histological details.

Fig. 3. Tangential slice from the wall of the spheroid similar to the section removed from the front surface of the wall of the secondary vesicle in the foregoing figure.

Fig. 4. Longitudinal section through an otocyst to show the concentric rings within the otolith.

Fig. 5. A spheroid with a portion of the wall of the secondary vesicle removed.


5
pp
sp
sv
o
c
s
2
4
su
ex
m
3
sl
r
en
nc
n
v
ec
o
1

A SYMBIOTIC FUNGUS OCCURRING IN THE FAT-BODY OF *PULVINARIA INNUMERABILIS* RATH.[1]

CHARLES T. BRUES AND RUDOLF W. GLASER.

During the late winter and spring of 1920 the present writers became interested in the supposedly symbiotic organisms which occur in various scale insects, hoping that they might be able to propagate some of them in artificial cultures and learn something of their physiological activities. From knowledge gained thus, it seemed probable that they might be able better to determine whether such organisms exist in the insects as true symbionts, as mere commensals or as innocuous parasites.

As is well known through the investigations of several workers, the entrance of the symbionts[2] into the egg of scale insects can be readily followed as well as their behavior during embryological development. A good account of this has been given by Shinji ('19) who also includes a summary of the previous work of other authors.

In the nymphal or full-grown scales of many species it is more difficult to find and interpret the symbionts and it seems probable that in some cases they must either become very much reduced in numbers, very highly modified, or perhaps reduced to minute and unrecognizable spores or granules.

Several workers who have recently examined the symbionts of Coccids (*e.g.*, Buchner '12, Sulk '10, Teodoro '18) regard them as yeasts (Saccharomycetes), although Berlese ('06) referred one species to the genus *Oöspora*, one of the *Hyphomycetes*.

Before beginning our work with *Pulvinaria innumerabilis*, the cottony maple scale, we examined several other species of Coccids, but were unable successfully to cultivate the symbionts from these, with the possible exception of one of the pine scales,

[1] Contribution from the Entomological Laboratory of the Bussey Institution, Harvard University, No. 176.

[2] We have used this name as a convenient designation already in current use and will discuss its appropriateness on a later page.

Chionaspis pinifoliæ Fitch. From the latter we isolated an organism, perhaps related to *Oöspora*, but we could not secure it with sufficient regularity to satisfy ourselves that it was really the symbiont, and not a contaminating species of microörganism of which one always encounters numerous species in work of this kind. Just how closely related the symbionts of various Coccids may be, must remain a matter of doubt until a considerable number have been carefully investigated, and preferably cultivated also, but our own observations lead us to think that more than one type of organism will be found after careful, systematic study.

In the following brief review of literature we have considered only such contributions as appear to bear directly on symbionts quite probably closely related to the form with which we have worked.

The first reference to the occurrence of symbiotic organisms in Coccidæ is that of Leydig ('54) who found discrete, lanceolate bodies which he believed to lie free in the lymph of *Coccus* (now *Lecanium*) *hesperidum*. He described them as 4 μ in length, multiplying by buds which do not separate till they have attained the size of the parent cell. Neither at this time, nor in 1860 in his contribution to the development of the Daphnids, did he realize their significance. From the rather large size and method of multiplication, these are probably similar to the organisms found in *Pulvinaria*, which also appear in freshly mounted smears as though they were free in body fluid. Putnam ('79) studied in considerable detail the biology, anatomy and development of *Pulvinaria innumerabilis* in Iowa. In connection with a section devoted to the contents of the ovaries he gave an account of the organisms with which we deal in the present paper. His observations were so carefully made that we have thought it worth while to include the following resumé. On opening a female at any time from October to May, five classes of bodies are set free, all of them apparently associated with the development of the eggs. These are: First, a clear protoplasmic liquid; second, clear spherical globules 10 μ–30 μ in diameter, lighter than water, and not taking the ordinary aniline stains. He was undoubtedly cor-

rect in believing these to be yolk and fat globules. Third, exceedingly minute, apparently spherical bodies, heavier than water and staining with eosine. Putnam thought that these might be bacteria although he suggests that they may be comparable to the blood-disks [erythrocytes] of vertebrates or that they may be stages in the development of the fourth class of bodies which he next considers. That all of these suppositions are probably incorrect will appear from our account on a later page. Fourth, small oval bodies 3 μ–5 μ in diameter and 10 μ long and heavier than water. These represent the organism which we have studied but Putnam was unable to decide whether they were spermatophores or whether they corresponded to the pseudonavicellæ observed by Leydig in *Lecanium,* as had been suggested to him by Dr. E. L. Mark.[1] A fifth class of bodies observed were the small incompletely formed eggs.

There can be no doubt that Putnam found the fungus with which we have worked as his description and figures make this point very clear. As he found it in all cases, it is further clear that the symbiont enjoys a wide range since his observations were made on specimens collected in the middle west and our own on material from eastern Massachusetts.

Metschnikoff ('84) found in a crustacean, *Daphnia magna,* a fungus which he called *Monospora bicuspidata.* This he regarded as a parasite, but recent developments in the study of apparently similar organisms in insects, suggests that these Crustaceans should be reëxamined.

Moniez ('87) described a fungus which he called *Lecaniascus polymorphus,* occurring in the scale insect, *Lecanium hesperidum.* He refers to Leydig's 1854 paper, mentioning the fact that *Lecaniascus* is evidently the same as Leydig's pseudonavicellæ. Moniez speaks of the organism as a parasite, and he found it in all specimens, both young and old, of the *Lecanium* that he examined. He described the isolated cells as 4–5 μ in length, and found mycelia attaining a length of 50–60 μ. Some doubt is cast upon this author's conclusions by Vejdovsky ('07) who suggests that Moniez may have seen two microörganisms, one represented

[1] Mark ('77) does not consider these bodies, however, in his paper on the anatomy and histology of the Coccidæ.

by the single cells and another by the mycelia and asci, the latter perhaps *Alternaria tenuis,* a parasitic fungus that attacks various Coccids of the genus *Lecanium.*

Lindner (1895) found in an European scale insect (*Aspidiotus nerii*) a yeast-like organism which he regarded as related to *Saccharomyces apiculatus.* By crushing one of the insects between a slide and cover-glass he observed large numbers of the yeasts, both between the small masses of fat-body and actually in the adipocytes. The organisms he described and figured as usually very long, pointed at one end, or lanceolate, sometimes joined in pairs by their acute tips, and frequently budding after the manner of yeast cells. At that time Lindner was unable to cultivate the organism although he evidently regarded it as a parasite, naming it *Saccharomyces apiculatus,* var. *parasitus.* He found it forming a mass at the posterior pole of the egg and made an ingenious explanation for its presence there, suggesting that one of the pointed tips perforated the egg and gave off a bud which then multiplied to form the mass or mycetome.

A later paper by Lindner ('07) which appeared in the *Wochenschrift für Brauerei* we have not seen, but from published reviews of this, it appears that it contains nothing bearing on the physiology or systematic position of his yeast-like organism.

Berlese ('06) studied in detail an organism which he found in *Ceroplastes rusci,* and was successful in growing it on artificial media. From Berlese's account, it appears that the fungus, which he calls *Oöspora saccardiana* is very similar to the one described by us in the present paper. The yeast-like cells in the Coccid vary in length from 4–12 μ, sometimes attaining a length of 16–18 μ in early summer, agreeing in size and also in form with those we have observed in *Pulvinaria.* In culture there is a great similarity in the general morphology and development of mycelia, and although Berlese gives few details concerning cultural characteristics, at least one statement shows a striking difference between the two. He found that although the symbiont from *Ceroplastes* grows rapidly on gelatine media, that these are not liquified, while as will appear from our account, the *Pulvinaria* fungus exhibits a powerful liquefactive action on gelatine.

Concerning the distribution of the symbionts in the body of *Ceroplastes,* Berlese gives no account, except to state that they generally occupy the visceral cavity completely in all individuals, in numbers estimated at from 60,000–70,000 cells.

Two other genera of soft scales, *Kermes* and *Physokermes,* have been the subject of investigation by Sulc ('07) and Vejdovsky ('07). The former found two distinct symbiotic organisms in two species of these Coccids, readily distinguishable from one another on the basis of size and form. These he described as representatives of a new genus, *Kerminicola.* Vejdovsky regarded them as Saccharomycetes in which opinion Sulc concurred. The microscopical structure of the symbionts was carefully described and is similar to that of the species in *Pulvinaria* studied by us, although *Kerminicola* evidently has a much more prominent and discrete nucleus and fewer multinucleate cells; also the form of the cells is generally much more elongate. Vejdovsky found the symbionts in the fat cells of the host in large numbers and observed them freed in the hæmolymph as a result of a disintegration of the adipocytes which he believed due to the activity of the included organisms. He, therefore, regarded them as parasites, but pointed out that their activities do not affect the gonads of the host, nor the development of the eggs in its body. They do, however, serve to break down the fat and to consume the remaining tissues of the host's body, after which it remains a shell for the protection of the now fully developed nymphal *Kermes.*

In a later paper, Sulc ('10) gives a more extended account of the similar organisms of a number of Homoptera and speculates at considerable length upon the relations between the insects and fungi. After more extended study, his ideas have been considerably modified and he has come to regard the microörganisms as essentially symbiotic in their association with their host insects. He suggests that the production of enzymes by yeasts (for he still refers the symbionts to the Saccharomycetes) must be considered in any interpretation of their physiological relations to the insects. Apparently he made no attempts at cultivation *in vitro.*

Pierantoni has considered the symbionts of certain Coccidæ in

several papers of which that of 1910 in the *Zoologischer Anzeiger* is of greatest interest in the present connection. In *Icerya purchasi,* he traced the entrance of the organisms into the egg of the scale insect and its subsequent behavior through the formation of the polar mass in the egg to the development of the mycetocyte in the larva. He found that the individual symbionts of this species were at first round or oval, and not noticeably elongated, and that later during embryonic development and at the time of hatching they became quite inconstant in form, varying from rounded or oval to much elongated and frequently strongly curved cells, all, however, of about the same diameter. These long forms may fragment, each piece becoming a new individual, while the short ones commonly divide by fission into equal parts. Occasionally, however, Pierantoni found cells multiplying by budding in the body cavity of the host, and more rarely in the mycetome.

On a gelatine medium, with high sugar content (20 per cent. saccharose) he was able to cultivate a yeast-like organism from the mycetocytes. These developed after four days' incubation as colonies that are described as small spheres in the gelatine which develop a sort of finger-like process which becomes prolonged toward the surface of the gelatine and then emerges projecting above it in the form of a finger, or with the base enlarged and pyriform. The individual organisms were of yeast-like form with buds more or less developed. It thus appears that the organism obtained in culture by Pierantoni differed greatly in form, size, and method of multiplication from the organisms in the insects. as will be shown later, the cultures obtained by us from *Pulvinaria innumerabilis* exhibit no such remarkable distinctions, in morphology and reproduction, from the cells in the host insect. Although he makes no mention of the development of mycelia in his cultures, the form of the colonies indicates without question that such must have been present, and that if the organisms in the mycetome were actually those obtained in culture, the symbiont of *Icerya* is a true fungus.

The Symbionts of the Half-grown Pulvinaria.

In early April the overwintered cottony maple scale-insects are partly grown and may be found attached to the bark of small twigs on the food plants, which consist of various maples and a few other woody plants. At this time they are nearing the end of their period of hibernation and do not yet exhibit any active growth.

If a specimen in this condition be crushed on a slide in normal salt solution, the symbionts may be readily seen free in the liquid. They are heavier than the medium and fall next to the slide, thus separating from the released fat globules which accumulate above, against the cover-slip. In such a preparation all the organisms appear to be in the liquid, as those in the fat cells are not readily discernible on account of their hyaline nature. This, no doubt, accounts for the statements that the symbionts occur in the lymph rather than in the tissue.

In sections it is evident, however, that the organisms are absent, or at least very nearly so, from the blood and that they are very generally distributed through the fat body, imbedded in the adipose cells. They are usually spaced in a quite regular way showing that they migrate or at least change their position in the cells subsequent to multiplication. The density of distribution is well indicated in the drawing (Fig. 1, *A*) which is made from a section of 6 μ in thickness where all of the symbionts present have been sketched. The photograph on Plate I., Fig. 1, is made from a typical cross-section through an entire insect, in which the symbionts appear as minute oval dots. On Plate I., Figs. 2, 3 and 4, are reproduced several small areas of the fat-body viewed at higher magnification with their symbiont inclusions. Sketches of a few still more highly magnified symbionts are shown in Fig. 1, *B*. Here it will be seen that they are extremely variable in size and shape, but always quite distinctly oval in form with one pole more acute and the opposite one more rounded. They vary in length from 10–16.7 μ by 5–6.5 μ in width, with an average size of 10–12.5 μ by 5.7 μ. Budding forms are frequently present, the bud developing at the narrow pole and separating either as a small oval, or more rarely rounded, cell. The buds at the time of sepa-

ration are of the same general shape as the cells from which they originate, but much smaller, varying in length from 6.2–6.7 μ. Some buds are nearly round, in which case they separate when considerably smaller, about 3.7 μ in diameter.

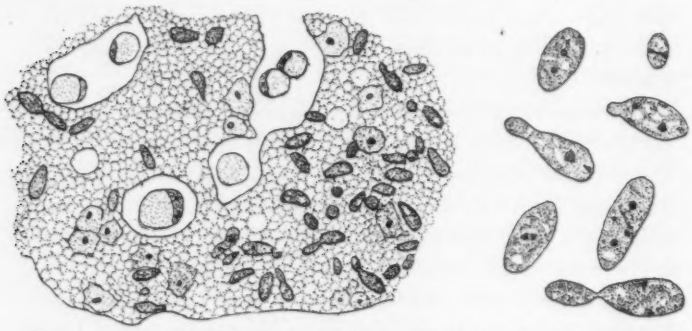


FIG. 1. *a*, Section of fat body showing symbionts *in situ* in overwintered nymphal *Pulvinaria*, × 260; *b*, isolated symbionts, stained with methylene blue and eosin, × 1,000.

The internal structure of the symbionts shows very little uniformity. Sections of tissue fixed in Zenker's solution and stained with methylene blue and eosin show one or several rather distinct deeply stained portions that resemble nuclei. When one is present it is usually central, when two or more are present, they are separated rather evenly from one another and from the cell wall. The remaining protoplasm shows irregular denser streaks and spots of irregular size, with usually one large, several smaller, or one large and one small vacuole, with generally a number of minute clear spots that can be seen only after very close examination. We have tried to bring out other details of structure by different methods of fixation and staining but without much success. Smears fixed by such methods as drying and submersion in absolute alcohol and subsequent staining with Giemsa's stain, Manson's Protozoan stain and carbol-fuchsin, give quite similar pictures to those in sections which are disappointing from a cytological standpoint. Evidently the symbionts are of very delicate consistency. No chains of symbionts occur at this time (early April) except for the single attached buds, and no masses of contiguous ones are to be found.

Changes in the Symbionts after Hibernation.

Our observations on these are very fragmentary as our available time at this season was occupied with the cultural experiments described below. It appears quite certain, however, that the oval symbionts of the early spring *Pulvinaria* nymphs undergo further multiplication and morphological changes in late April, and during May. A cursory examination of specimens about May 10 showed the symbionts in groups sometimes forming short strings, somewhat similar to the typical mycelium which was obtained in cultures. The picture at this time indicates quite clearly the fungous nature of the yeast-like organisms just described, found before spring growth begins.

Isolation of the Fungus from Pulvinaria.

Our first attempts to cultivate in artificial media the yeast-like cells present in *Pulvinaria* were made in early April. The overwintered, partly grown scales were brought into the laboratory, still attached to the maple twigs on which they occur. The scales can readily be detached from the twig by means of a sterile needle and allowed to fall upon a sterile microscope slide. The scales thus removed, were treated in several ways as experience had taught us that material of this sort is very apt to be contaminated on the surface by various microörganisms. Some were treated with 85 per cent. alcohol for a few minutes, others rapidly passed through the flame of a Bunsen burner, and others were used without treatment, except to avoid contact with any unsterile object. Our first media were potato agar and "sugar agar" which consisted of potato agar to which varying amounts of maple sugar (2½ per cent.–20 per cent.) had been added. These tubes are readily inoculated by crushing the scale insect between two microscope slides and streaking the agar with a loop dipped in the body-juices thus extracted. It is also easy to drop the whole insects into culture tubes and then crush them on the agar by means of a dissecting needle. From a large series of tubes inoculated according to these methods, nearly one half showed after three days a good growth, white in color, spreading on the surface of the media. These colonies appeared to de-

velop almost equally well in the greatly diverse concentrations of sugar and in the plain potato agar. A microscopic examination at this time showed that nearly all the tubes in which growths occurred contained the same microörganism, and that only a few were contaminated by molds (*Penicillium*) and bacteria. The abundant species showed large numbers of budding yeast cells like those in the living scale insects and the development of mycelium as well showing that the symbiont was a fungus and not a yeast as one might otherwise be led to believe from a study of the living insects, at this season of the year when only single budding cells occur in the fat body.

Several of the colonies thus obtained were plated, found to be pure and sub-cultures were then made from these which have furnished the material for the description and cultural characters detailed below. Although the morphology of the organism in the living insects and in the cultures and the fact that it was recovered in such a large proportion of the cultures, left little doubt as to the identity of the two, we undertook some serological tests to corroborate if possible the conclusion based on morphological data.

For this purpose, two rabbits were secured, inoculated with bouillon cultures of the fungus, and serum from each was tested with the cultures and also with the organisms in the living scale insects. The following table shows the doses used and the reactions of the rabbits.

Date.	Amount of Culture.	Weight.	
		Rabbit A.	Rabbit B.
April 13	3 c.c.	2185 gms.	1905 gms.
April 16	5 c.c.	2180 gms.	1910 gms.
April 20	5 c.c.	2080 gms.	1910 gms.
April 24	6 c.c.	2110 gms.	1895 gms.
April 29	6 c.c.	2030 gms.	1915 gms.

One week later some blood was withdrawn from each rabbit and serum prepared. A precipitin test with the culture gave a positive reaction after four hours, and agglutination was very pronounced after one and one half hours as examined under the microscope. On account of the impossibility of securing sufficient material from the living scales, it was possible to try with

them only the agglutination test. With these the reaction was not so pronounced as with the yeast-like forms in culture, but nevertheless distinctly positive. Unfortunately by the time the animals had been immunized (early May) the number of yeast-like cells in the insects had decreased and the reaction could not be so readily observed as in the cultures, or so well as it might have been several weeks earlier in the spring when the insects contained innumerable, separated, oval cells. The data from the rabbit experiments has, however, convinced us that there can be practically no question that the organism cultivated is actually the one present in the insects. Furthermore, since we have never failed to observe it in living scales from this locality, and since Putnam (v. *antea*, p. 301) found it invariably present in Iowa, it is undoubtedly present regularly in *Pulvinaria innumerabilis*.

Cultural Characteristics of the Fungus.

As stated at the outset, we wished to grow the symbiont on artificial media, not only to describe it adequately, but to determine as completely as possible its physiological activities. In order to do this, sub-cultures from the original isolations were planted upon various media, such as are in general use by bacteriologists and mycologists. From these, the following observations were made.

Growth on Solid Media.

Potato Gelatine Colonies.—Growth rapid; after 72 hours, cottony with flocculent elevated center and filamentous edge, diameter of center 1 mm., width of margin 1 mm. Liquefaction cup-shaped.

Nutrient Gelatine Colonies.—Growth slow; after 72 hours, rounded, with central elevation. Diameter 0.3 mm., with roundly lacerated edge. Liquefaction cup-shaped.

Potato Agar Colonies.—Growth rapid; after 72 hours, filamentous, ciliate (sub-surface) or rounded (surface). Disk when present, smooth; elevation convex; edge of round colonies smooth, that of irregular colonies radiately filamentous or ciliate. Internal structure finely granular. Diameter 1.5–2.5 mm.

Nutrient Agar Colonies.—Growth slow; after 72 hours, round,

with smooth surface and convex elevation. Edge smooth; internal structure finely granular, with irregular central core. Diameter 0.6 mm.

Potato Gelatine Stab.—Liquefaction begins in 48 hours; growth best at tip, the line of puncture filiform; liquefaction at first napiform, becoming stratiform, surface umbonate. Medium unchanged.

Nutrient Gelatine Stab.—Same, but liquefaction proceeds more slowly and is more nearly crateriform.

Potato Agar Slant.—After 72 hours growth is abundant, spreading, densely rhizoid, convex. Color white, opaque, surface glistening, smooth. No odor, consistency viscid. Medium unchanged.

Nutrient Agar Slant.—After 72 hours growth is moderate, beaded, with a few rhizoid colonies where medium is thin. Surface smooth, glistening. Color white, opaque. No odor, consistency butyrous. Medium unchanged.

Locke's Agar Slant.—Growth like other agar slants, scanty, beaded, many rhizoid colonies, consistency butyrous. After a much longer incubation (four weeks) there is not a very heavy growth, but it is still more or less beaded and highly rhizoid on the sides and extending deep into the agar.

Molisch's Agar Slant.—After 72 hours growth abundant, much like that on potato agar. Consistency very ropy. No pigment, even in old cultures.

Potato Agar (with Yeast[1]*) Slant.* Growth after 72 hours abundant, like that on potato agar, but with fewer rhizoids.

Potato (Pieces).—Colonies raised, slightly yellow, growth good. There is a trace of ammonia.

Growth in Liquid Media.

Nutrient Bouillon.—After 48 hours growth good; after 72 hours, no surface growth, no clouding and no odor, but with an abundant viscid sediment. No hydrogen sulphide is produced.

Locke's Solution.—After 48 hours not very much growth; after 72 hours, no surface growth, no clouding and no odor, but with an abundant viscid sediment. After a much longer incuba-

[1] Made by adding 2 per cent. of thoroughly crushed bread yeast.

tion at room temperature (four weeks), the growth becomes very flocculent and adheres to the surface of the glass where it is finely dotted with very dark, pigmented spots.

Molisch's Solution.—After 48 hours rather good growth; after 72 hours, no clouding or surface growth, but with very abundant, slightly viscid sediment, no odor.

Sugars.—We have grown the organism in six sugars: lactose, dextrose, mannite, saccharose, levulose and maltose. None of these, however, furnish any differentiating characters; in all there is good growth without the formation of gas, but with heavy viscid sedimentation, and after prolonged incubation, the development of a distinct surface film.

Milk.—Growth is abundant, and after four to six days incubation the milk begins to clear at the top, sediment collecting in the lower half of the liquid. Litmus milk becomes distinctly red at the time of clearing. After about a week, the liquid becomes whey, with a sediment at the bottom of the tube.

Anaërobic Media.—We have not been able to cultivate the organism under anaërobic conditions.

Production of Enzymes.

Protease.—Gelatine is rapidly liquefied. Milk cultures, tested after 20 days, give a positive reaction with $MgSO_4$, NaOH and $CuSO_4$, showing the presence of peptones.

Lipase.—After both 20 and 30 days' incubation, cultures in either whole or skimmed milk give positive reactions. There is a strong odor of butyric acid. Ethyl butyrate is also decomposed with the formation of butyric acid. The 30-day culture was tested with pyrogallol and stannic chloride and gave a positive reaction also.

Diastase.—Bouillon cultures after ten days' incubation were treated with starch paste at incubator temperature for 48 hours; after this, Fehling's solution was reduced, demonstrating the presence of sugar.

Morphology of the Cultivated Fungus.

The cultures show during the first few days only yeast-like budding cells like those seen in the early spring in the fat-body

of the insects. These vary considerably in size, ranging from 6–16 μ in length and from 3–9 μ in width. They are thus more variable in size in culture than in the insect and generally more elongate. The maximum length is about the same, but there are more smaller cells in culture, due no doubt to the fact that during rapid development the buds separate when less fully developed than in the insect. The internal structure when stained, appears to be the same as that of the forms in the fat-body described above.

After prolonged incubation on solid media the formation of a distinct mycelium always occurs. This is at first white, but after several weeks, blackened spots sometimes become visible, due to the development of pigment in the walls of certain groups of cells. This occurs especially on potato-agar. In at least one liquid medium, Locke's solution, the same blackened cells develop.

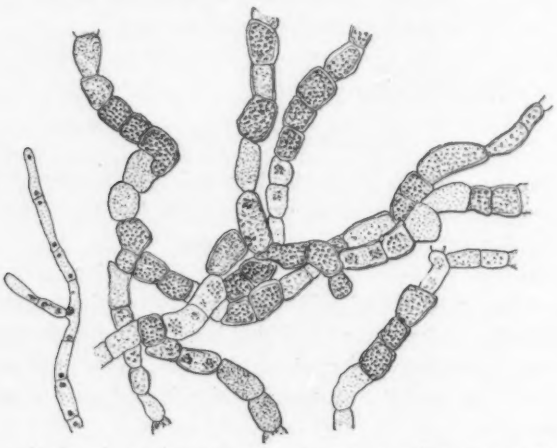


FIG. 2. Portion of mycelial growth of symbionts after prolonged incubation (10 days) in liquid bouillon medium. Magnified about 400 diameters.

The mycelium (Fig. 2) is branched and of quite irregular form. The larger hyphæ measure from 6–15 μ in diameter, broad and narrow cells frequently alternating or with one size interpolated in series of the other. Some cells, usually single ones or pairs, more rarely several in succession are heavily pig-

mented and appear very dark in fresh material; a pair of these are frequently rather closely fused to form an oval body. The contents of these dark cells are highly granular, with the protoplasmic mass clearly separated from the cell wall by a hyaline layer. Toward the tips the hyphæ are usually much more slender, pale in color and with only scattered granules in the protoplasm. The method of branching is entirely dichotomous, many lateral branches along the larger hyphæ consisting of but a single cell although both near the tips and on the larger hyphæ there are many long branches which again subdivide. Very rarely there is an anastomosis of the finer apical branches formed by lateral prolongations of the cells. In preparations we have had difficulty in recognizing the conidiophores, but free conidia are present in old cultures. They measure from 8–10μ in length and are broadly oval in form, very nearly transparent and without color.

Systematic Position of the Cultivated Fungus.

We have been unable to deal with this matter in a satisfactory way owing to our unfamiliarity with cryptogamic botany and must leave it for consideration by an experienced mycologist. It is very evident that the symbiotic organism in *Pulvinaria* cannot be regarded as a Saccharomycete, although its morphology and method of multiplication in the insect does not preclude such an assumption. Indeed, the symbionts that have been observed in other Coccids and in most other Homoptera as well have usually been regarded as yeast-like organisms and commonly referred to the genus Saccharomyces or to new genera located in the same group of plants. Berlese ('06) has placed the organism which he cultivated from *Ceroplastes* in the genus *Oöspora*, thus recognizing it as a true fungus, but hitherto, with the possible exception of Pierantoni ('10) no one else seems to have been successful in growing *in vitro* any of the symbionts of coccids.

The species which we have obtained from *Pulvinaria* seems to be quite similar to the one described and figured by Berlese from *Ceroplastes* so far as the general morphology of the yeast-like cells in the coccid and the mycelial structure in culture. Neither species, however, has been sufficiently studied to make a more posi-

tive statement. Dr. O. F. Burger kindly examined some of our cultures and has expressed the opinion that they probably represent a species of *Dematium* or a related genus. Such morphological characters as we have been able to make out agree well with descriptions of this genus to which it may be tentatively referred.

Physiological Rôle of the Symbiont.

As stated at the outset, we have attempted to determine the physiological behavior of the *Pulvinaria* symbiont in culture to ascertain in what way it may affect the metabolism of the coccid.

Contrary to what occurs in the case of most yeasts, this organism produced no gas in media made from any of the sugars in which it was grown. This is quite what might be expected as the coccid tissues are undoubtedly rich in sugars and any organism producing gas in the presence of such substances could not be tolerated in the body of the coccid.

On the other hand a diastatic ferment is produced in quite appreciable quantities. Whether this bears any relation to the metabolism of the coccid is not entirely clear. In the adipose tissue and body liquids, starch is probably not present to any considerable extent, although in the large quantities of plant sap ingested by the coccids there must be substances upon which this ferment might act. It has been shown also by Büsgen ('91) that certain modifications are produced in the tissues of the food plants of Coccids at the point where the mouth setæ are thrust into the plant. These modifications appear to be induced by secretions actually injected into the plant tissue by the insects and they may act in liquefying or in partly digesting already liquid or semi-liquid material, before it is withdrawn by the insect. It is quite possible therefore that a diastatic ferment might act in two possible ways in aiding the digestion of the coccid. If freed in the blood, it might either pass into the alimentary tract, there to act upon ingested food, or it might be taken up by the salivary glands to be later injected into the plant and thus act as an extra-intestinal digestive agent. Such extra-intestinal digestion is known to occur in several diverse insects, although in these cases the ferments are no doubt elaborated directly by the salivary glands.

We have also clearly shown that a proteolytic enzyme and a lipase are produced abundantly by the *Pulvinaria* symbiont. The possibilities for these to influence the digestion and metabolism of the coccid are more diverse than those presented by the presence of diastase. Both, particularly the lipase, must act upon the adipose cells in which the symbionts occur. We might therefore suppose that they assist in the rapid breaking down of this tissue at the time of maturity when the eggs of the *Pulvinaria* are rapidly developed. That they may assist in digestion, either in the body or through the agency of secretions injected into the plant is also quite possible, although such indirect action must undoubtedly be secondary if it occurs at all.

The General Nature of the Relation between Symbiont and Coccid.

The symbionts have gradualy come to be regarded rather generally as truly symbiotic organisms, although those who first studied them naturally assumed that their presence indicated some sort of parasitism. There are several reasons why it is difficult to believe that they are actually parasitic. In the first place, not only in *Pulvinaria*, but in the other species in which they have been found, they are universally present in all the individuals of a species in approximately equal numbers. Many true parasites, *e.g.*, certain Nematode worms, the Protozoan parasites of human malaria, etc., commonly appear with great frequency in the bodies of their hosts, but their occurrence never includes all the individuals of a host species, except at certain times and places where parasitism is unusually heavy and assumes the form of an epidemic. In such cases also, the affected population is not in a healthy condition and species so generally affected cannot be expected to represent ones well fitted to survive and become abundant. *Pulvinaria* and other Coccids certainly cannot be placed in such a category. On the other hand the presence of detrimental parasites results in tissue changes or disturbances of metabolism that can be recognized. Such can be seen in the behavior of the fat-body in *Pulvinaria* and other genera (Sulc, '11), but as has just been said this is most readily regarded as beneficial rather

than detrimental as the fat is not broken down until the time that it would normally disintegrate to supply nourishment for the developing eggs of the coccids.

Without any definite indication of pathological changes, it seems impossible, therefore, to regard the universally present symbionts as harmful parasites.

It has also been suggested that they may represent innocuous or indifferent parasites and it is not so easy to distinguish between these and true symbiotic or benign organisms from their effect on the coccids. As a matter of fact it seems necessary to regard all three as steps in an evolutionary process, harmful parasites in their first association, later as innocuous ones and finally as true symbionts. These will follow one another as the host adapts itself to withstand or nullify any ill effects of the parasite until it finally is able to utilize the products of the intruder to further its own metabolic processes.

Thus it seems reasonable to regard these three types of association as not clearly distinct from one another, but as connected by intergrades.

Since, however, there is good reason to believe that the production of diastase, protease and lipase by the symbionts may serve to benefit the coccids, the possibility of real symbiosis cannot be excluded.

There is one point, however, which needs further study. By a minute study of the changes in the tissue of the food-plant adjacent to the proboscis of the feeding Coccid, it should be possible to gain much additional evidences upon the changes which undoubtedly occur in such tissue. This we have not had opportunity to undertake. Why the disintegration of the fat-body is delayed till the proper time in the life-cycle of the coccid also is not clear. Since, however, changes in the vegetative character of the symbiotic fungus are initiated in the late spring, it seems probable that they may determine to some extent the quantities of enzymes produced. On the other hand it is evident that the coccid is able to inhibit any excess development of the symbiont as the number of symbiont cells remains very uniform and never seems to increase beyond certain bounds, quite a different condi-

tion from that obtained among pathogenic parasitic microörganisms.[1]

This indicates a nice physiological balance between the coccid and symbiont and is another reason for considering this a case of true mutualism.

BIBLIOGRAPHY.

Berlese, Am.

'06 Sopra una nuova specie mucidinea parassita del *Ceroplastes rusci*. Redia, Vol. 3, pp. 8–15.

Breest, F.

'14 Zur Kenntnis der Symbiontenüberträgung bei viviparen Cocciden und bei Psylliden. Arch. f. Protistenk., Vol. 24, pp. 263–276, 2 Taf.

Buchner, P.

'11 Ueber intrazellulare Symbionten bei zuckersaugenden Insekten und ihre Vererbung. SB. Ges. Morph. Physiol. München, Vol. 27, pp. 89–96.

'12 Studien an intracellularen Symbionten. I. Die intracellularen Symbionten der Hemipteren. Arch. f. Protistenk., Vol. 26, pp. 1–116, Taf. 12.

Büsgen, M.

'91 Der Honigtau, biologische Studien an Pflanzen und Pflanzenläusen. Jenaische Zeitschr. Naturwiss., Vol. 25, pp. 339–428, Taf. 2.

Howard, L. O.

'00 The Two Most Abundant Pulvinarias on Maple. Bull. Div. Entom. U. S. Dept. Agric., n. s., No. 22, pp. 7–16.

Leydig, F.

'54 Zur Anatomie von *Coccus hesperidum*. Zeits. wiss. Zoöl., Vol. 5, pp. 1–12, Figs. 6

Lindner, P.

'95 Ueber eine in *Aspidiotus nerii* parasitisch lebende Apiculatus-hefe. Centralbl. f. Bakt., Vol. 1, pp. 782–787, Figs. 9.

'07 Das Vorkommen der parasitischen Apiculatus-hefe. Wochenschr. f. Brauerei.

Mark, E. L.

'77 Beiträge zur Anatomie und Histologie der Pflanzenläuse, insbesondere der Cocciden. Arch. Mikr. Anat., Vol. 13, pp. 31–86, Taf. 3.

Mercier.

'07 Recherches sur les bactéroïdes des blattides. Arch. f. Protistenk., Vol. 9, pp. 346–358, Taf. 2.

Metchnikoff, I.

'84 Ueber eine Sprosspilzkrankheit der Daphniden. Arch. f. path. Anat. u. Physiol. u. f. klin. Med., Vol. 96, p. 177.

Moniez, R.

'87 Sur un champignon parasite du *Lecanium hesperidum* (*Leciniascus polymorphus nobis*). Bull. Soc. Zoöl. France, Vol. 12, pp. 150–152.

[1] In this connection it is interesting to note that we found antibodies developed abundantly in rabbits immunized against our cultures of the *Pulvinaria* symbionts.

Pierantoni, U.

'09 L'origine di alcuni organi della *Icerya purchasi* e la simbiosi ereditaria. Bull. Soc. Napoli, Vol. 23, pp. 147–150.

'10a Origine e struttura del corpo ovale del *Dactylopius citri* e del corpo verde del *Aphis brassicæ*. Boll. Soc. Nat. Napoli, Vol. 24, pp. 1–4.

'10b Ulteriore osservazione sulla simbiosi ereditaria degli Omotteri. Zoöl. Anz., Vol. 36, pp. 96–111, Figs. 10.

'12 Studi sullo sviluppo d'*Icerya purchasi* Mask. Part I. Origine ed evoluzione degli elementi sessuali femminili. Arch. Zoöl. Ital., Vol. 5, pp.

'13a Studi sullo svlluppo d'*Icerya purchasi* Mask. Part II. Origine ed evoluzione degli organi sessuali maschili. Arch. Zoöl. Ital., Vol. 7, pp. 243–274, Tav. 3.

'13b Struttura ed evoluzione dell'Organo simbiotico di *Pseudococcus citri* Risso e ciclo biologico del *Coccidomyces dactylopii* Buchner. Arch. f. Protistenk., Vol. 31, pp. 300–316, Taf. 3.

Putnam, J. D.

'80 Biological and Other Notes on Coccidæ. Proc. Davenport Acad. Sci., Vol. 2, pt. 2, pp. 293-347, 1 pl.

Sanders, J. G.

'05 The Cottony Maple Scale. Circ. Bur. Entom., U. S. Dept., n. s., No. 64, pp. 6.

Shinji, G. O.

'19 Embryology of Coccids, with Special Reference to the Ovary, Origin and Differentiation of the Germ Cells, Germ Layers. Rudiments of the Mid-gut, and the Intracellular Symbiotic Organism. Journ. Morphol., Vol. 33, pp. 73–167, Pls. 20.

Sulc, K.

'07 *Kerminicola kermesina* n. gen., n. sp., und *physokermina* n. sp., neue Mikroendosymbiotiker der Cocciden. SB. kgl. böhm. Gesellsch. Wiss. Prag., Jahrg. 1906, art. 19, pp. 6, Figs. 2.

'11 Pseudovitellus und ähnliche Gewebe der Homopteren sind wohnstätten symbiotischen Saccharomyceten. SB. kgl. Böhm. Gessellsch. Wiss. Prag. Jahrg. 1910, art. 3, pp. 39, Figs. 18.

Teodoro, G.

'12 Richerche sull'emolinfa dei lecanini. Atti Acad. ven.-trent.-istr., Anno 5, pp. 72–84.

'16 Osservazione sulla ecologia delle cocciniglie con speciale riguardo alla morfologia e alla fisiologia de questi insetti. Redia, Vol. 11, pp. 129–209, Figs. 3, Pls. 3.

'18 Alcune osservazione sui saccaromiceti del *Lecanium persicæ* Fab. Redia, Vol. 13, pp. 1–5.

Vejdovsky, F.

'07 Bemerkungen zum Aufsatze des Herrn Dr. H. Sulc ueber *Kerminicola kermesina*. SB. kgl. böhm. Gesellsch. Wiss. Prag, Jahrg. 1906, art. pp. 6–12, Fig. 1.

EXPLANATION OF PLATES.

PLATE I.

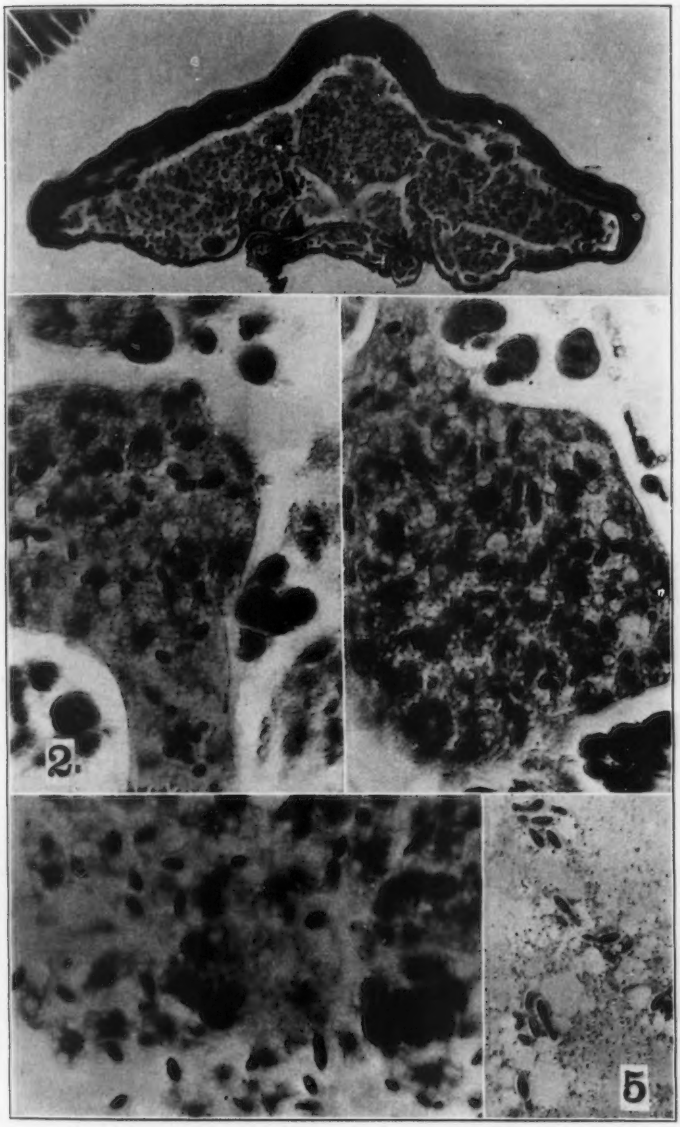
1. Cross-section of overwintering Coccid, showing distribution of symbionts in the fat-body. Low magnification.

2. Portion of fat-body at higher magnification with symbionts included in adipose cells. Magnification about 400 diameters.

3. Similar portion, showing more abundant symbionts. Magnification about 400 diameters.

4. Symbionts in adipose tissue which is apparently in process of disintegration. Magnification about 400 diameters.

5. Smear from culture of symbionts in liquid medium after 48 hours incubation. Magnification about 400 diameters.



C. T. BRUES AND R. W. GLASER.

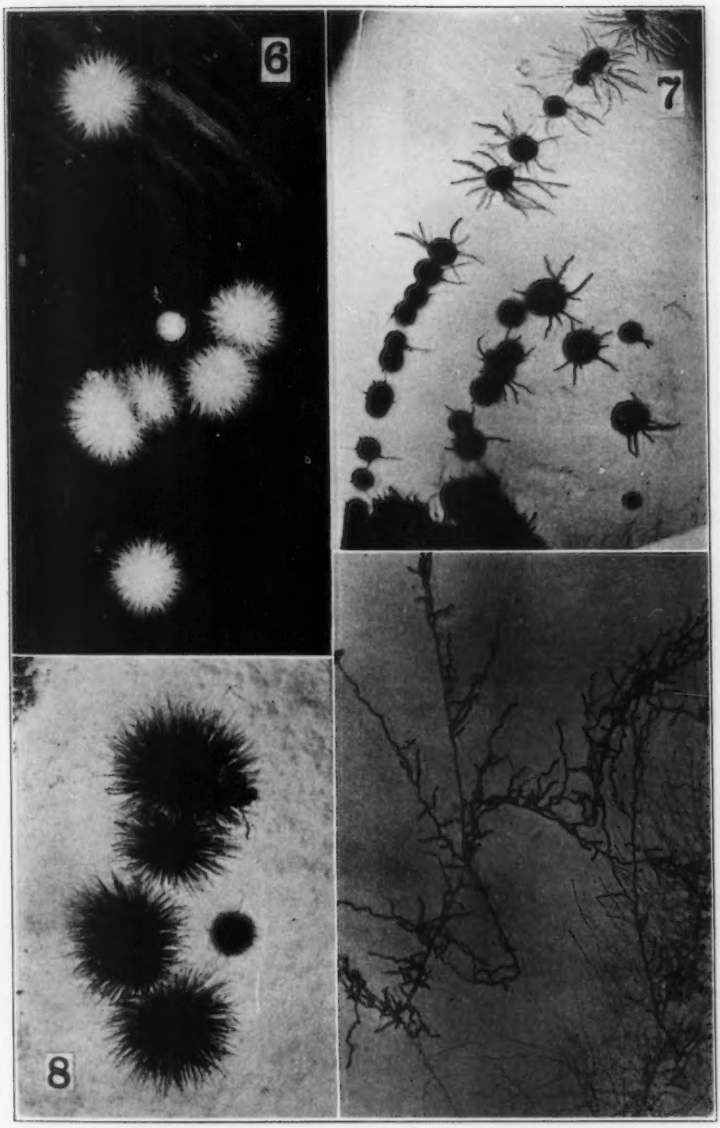
PLATE II.

6. Form of young colonies of symbiont after 72 hours incubation on potato agar plate, viewed by reflected light. Magnified 5 diameters.

7. Group of colonies of symbiont on nutrient agar after 10 days incubation. Magnified 4 diameters. Note small size of colonies and paucity of processes.

8. Group of colonies on the plate illustrated in figure 6, at same magnification, viewed by transmitted light, to show internal structure and manner in which radial processes develop.

9. Development of mycelia in liquid culture of symbiont after prolonged incubation of 10 days. Magnified about 60 diameters.

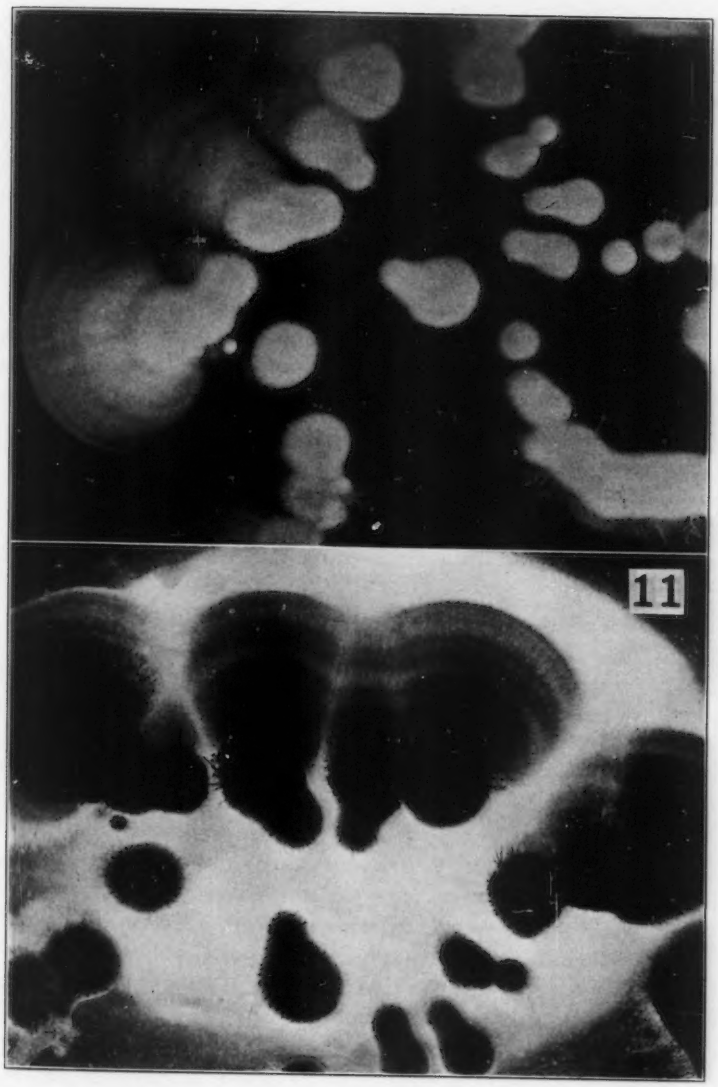


C. T. BRUES AND R. W. GLASER.

PLATE III.

10. Gross appearance of colonies of symbiont after prolonged incubation (12 days) on potato agar. Viewed by reflected light to show peripheral processes. Magnified 3 diameters.

11. Portion of same plate at same magnification, viewed by transmitted light, to show internal mycelial structure.



C. T. BRUES AND R. W. GLASER.

THE MIGRATION OF THE PRIMARY SEX-CELLS OF FUNDULUS HETEROCLITUS.

A. RICHARDS AND JAMES T. THOMPSON,

ZOÖLOGICAL LABORATORY, UNIVERSITY OF OKLAHOMA.

The origin of the primary sex-cells in vertebrates is a problem which has received considerable attention during late years. Extensive summaries of the literature upon this subject may be found in the articles of Allen, 1911, and Jordan, 1917. Since no complete agreement with regard to details has as yet been reached, it is perhaps desirable to review briefly the earlier investigations, and to point out any discrepancies in the results already obtained which would seem to require further study. It would seem that more evidence is necessary to warrant safe conclusions on the matter.

Waldeyer (1870) first described the differentiation of the sex-cells from the "germinal epithelium" of a four-day chick. His view of sex-cell origin from the mesothelium covering the mesonephros was accepted at the time, and has been supported even by recent investigators. In 1880 Nussbaum advanced a rival theory as a result of his observations on the embryology of the trout and frog. He held that the sex-cells were of blastomeric origin, and further that there was an extra-regional segregation and a migration to the germ gland. Weismann (1886) popularized this idea in his work on the "continuity of the germ plasm."

Since the time of Nussbaum the evidence against the "germinal epithelium" idea has steadily increased. A number of investigators (Hoffman, 1892; Eigenmann, 1892; Beard, 1900; Woods, 1902; Allen, 1906, 1907, 1911; Dodds, 1910; Swift, 1914, 1915, 1916; Jordan, 1917) have failed to find any conditions not in accord with Nussbaum's theory.

However other recent workers (Firket, 1914, 1920; von Berenberg-Gossler, 1914) have been unable to accept this interpretation of the activities of these cells. According to their viewpoint, the

migration of the primary sex-cells is reduced to a mere phylogenetic vestige and is without any great genetic significance. Firket speaks of the primordial germ-cells as "primary genital cells" which, after migration disintegrate in the germ gland, being replaced by the true of "secondary genital cells" which arise from the peritoneal cells of the germ gland. Von Berenberg-Gossler regards them as mesodermal wandering cells of late endodermal origin, and describes them as contributory in the formation of the Wolffian ducts.

In our study of this general problem in *Fundulus* a number of questions have arisen as separate phases of the matter. The blastomeric origin of the sex-cells, their path and method of migration, and their history after reaching the germ gland are all matters requiring separate study. This paper has, as its special aim, the definite identification of the primary sex-cells and the determination of the germinal path in *Fundulus* embryos; that is, it is concerned with the second question listed. As yet our data upon the first question is inadequate and we have not enough material for a study of the third.

Material and Methods.

The material for this investigation consisted of the eggs of the teleost, *Fundulus heteroclitus* and was collected at Woods Hole in the summer of 1919. Care was exercised to insure an approximately uniform fertilization of the ova by mixing them with chopped testis. Two extensive series were preserved during the summer. Although accurate records were kept as to the age of each group, they are of only nominal value in this investigation, since environmental and individual differences cause variations in development of embryos of like age.

All embryos in these two series were fixed in Bouin's fluid and stained by the familiar "long method" for iron hæmatoxylin. Other material in various fixatives was also available for comparison. No trouble was experienced in obtaining slides which show clearly the cytological characteristics throughout the series as far as described. A majority of sections were cut 4 micra thick, but some were cut 5, 6 and 7 micra. The thickness of all sections

was of course recorded. Most of the observations were made from serial transverse sections because they show the dorso-ventral position of the sex-cells more clearly in relation to the outstanding features of the developing embryo than do those cut longitudinally.

Observations.

Criteria of the Primary Sex-cells.

The enumeration of criteria for any group of cells as distinguished from all others in a series of embryos is a task which promises but doubtful results. There can be no question however that the primary sex-cells do have distinctive characteristics which make them easily recognizable, during the resting stages, to one who has had them under observation. It is not always feasible positively to identify the cells during division.

Throughout the migration period these cells maintain the same general characteristics. There are, to be sure, slight variations in the ratio between the nuclear and cytoplasmic elements, in size and in the character and arrangement of the chromatin granules; but these features may be observed only on close inspection, rather than in a preliminary study of the primary sex-cells.

The primary sex-cells vary in diameter from 9 to 12 8 micra. As contrasted with other cells they are spherical or ovoid with very definite cell outlines. The nuclei conform to the general shape of the cell body within which they are located. The cytoplasmic content is always clearer and takes less stain than that of the surrounding cells. Likewise the achromatin of the nuclei is quite clear, allowing the chromatic granules to stand out in bold contrast. The linin network is directly beneath the nuclear membrane, and due to this arrangement the chromatin granules are distributed peripherally over the nucleus. This peripheral arrangement of the chromatin is a constant distinguishing characteristic not to be mistaken, for it is never produced in any other cells. The linin network is connected to one, or more frequently to two nucleoli which are located near the center of the nucleus. No peculiar invagination of the nuclear membrane, such as was reported by Dodds (1910), was observed in *Fundulus*. An unusually large centrosome is, as a rule plainly visible in the cyto-

plasm. In older embryos these cells may be recognized by their size, since they are larger than any others which may occur in the same region.

Figures 1, 2, 3 and 3*a* are surface views of typical sex-cells. The peripheral arrangement of the chromatin has been emphasized in drawing Fig. 4*b*, by focusing upon a level with the center of the nucleus. Fig. 4*a* was obtained by focusing higher on the surface of the same nucleus. Thus Fig. 4*b* represents the chromatin knots in an optical section; while Fig. 4*a* shows them in a surface view. If Fig. 4*a* were superimposed upon Fig. 4*b* the resulting composite would be a cell not unlike that represented in Fig. 3, except that in the latter the knots have taken the familiar granular appearance.

A positive identification of the primary sex-cells was first made in a 24-day embryo. From this stage their path was followed backwards, through all the intermediate phases of migration, until they were no longer evident. It is considered expedient to describe their position in the 24-day stage, so that no question shall arise later as to the exact nature of the migrating cells whose course is to be traced.

Having established the identification of the sex-cells in the late embryo (24 days) their migration may be traced from their earliest appearance up to this stage. Although this sequence is contrary to our experimental procedure, it is believed to be more easily followed by the reader.

24-Day Embryo. 5.75 Mm. Long.—At the 24-day stage the sex-cells lie in the sac-like anlagen of the germ glands, which have formed dorsally and slightly laterally to the hind gut. Here they are unquestionably recognizable (Fig. 5). These cells are numerically inferior to the peritoneal cells which surround them and which are beginning to take a very active part in the formation of the future sex gland. The size and position of the germ gland anlagen in relation to the embryo is shown in Figs. 6 and 7. No attempt has been made to ascertain the average number of sex-cells which are present during this stage.

Whether these are the true sex-cells as maintained by many investigators, or whether they later disintegrate and become replaced by "secondary genital cells" as indicated by Firket (1914,

1920) and others, is a question which may be omitted from the present discussion.

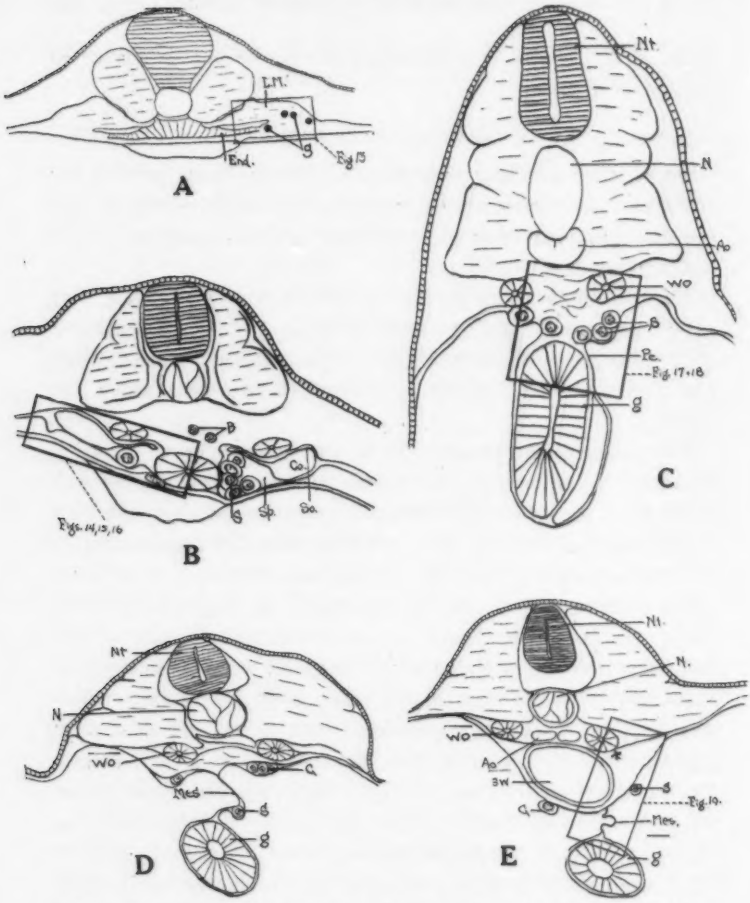
Observations of the Germinal Path.

From many available embryos the following were selected for consideration because they constitute representative stages, and are essential to a clear understanding of the migration of the primary sex-cells.

Embryos from 46 to 50 Hours.—Three embryos of this very early stage, designated in our material as B′ 21X, B′ 21L and B′ 23 respectively were carefully studied. Others were available but they were used merely as checks on the three which are reported.

The position of the sex-cells at this stage is most striking. There is a wide range of distribution in each embryo. The most anterior of the sex-cells were invariably farther along the germinal path than were the more posterior ones of the same embryo. To demonstrate this fact Fig. 20 has been drawn; it is an exact outline diagram of B′ 21X, reconstructed by the most accurate means possible. The lateral extent of the neural tube, of the mesoderm and the positions of the sex-cells were determined by measuring from the median line. The thickness of the sections was known. These determinations were plotted on millimeter paper and the outline filled in as indicated by the plotted guides. The exact antero-posterior positions of the sex-cells were determined by counting the sections of the serially sectioned embryo. Figs. 9, 10 and 11 are outline drawings from the embryo B′ 21X which was sectioned transversely, and Fig. 12 from B′ 21L which was sectioned longitudinally. These drawings show the exact positions of the sex-cells more clearly than would be possible in a written description.

The letters *A*, *B* and *C* on Fig. 20 indicate the positions of the sex-cells shown in Figs. 11, 10 and 9 respectively. Fig. 11 illustrates clearly the position of the primary sex-cell in the extra-embryonic region at the posterior of the embryo. Four sex-cells are shown in Fig. 12 as being lateral to the undifferentiated endodermal cell mass and ventral to the elongating tail. In Figs. 9 and 10 the migration has progressed proportionally to the devel-



TEXT FIG. *A*. Semidiagrammatic transection through the posterior of the 50-hour embryo B′ 23. The spots indicate the positions in which the greater portion of the sex-cells are found at this stage. The rectangle includes the area which is drawn in detail in Fig. 8. × 225.

TEXT FIG. *B*. Transection from the 105-hour embryo, showing the first decided advance over the stage illustrated in Text Fig. *A*. Here the gut, Wolffian ducts and the cœlome have taken form. The rectangle includes the area drawn in Figs. 14, 15 and 16. × 225.

TEXT FIG. *C*. Showing progress of development after 6 days. Figs. 17 and 18 are detailed drawings of the area included in the rectangle. × 225.

TEXT FIG. *D*. Transection through the developing gonads of the 9-day embryo, B 34. The sex-cells are collected ventral to the Wolffian ducts. The dorsal mesentery shows a decided change from conditions found in earlier stages. × 90.

TEXT FIG. *E*. From the 13-day embryo B 42, showing the effect of the developing swim bladder. × 90.

opment of the embryo at the regions represented. The sex-cells lie between the periblast and the endoderm in Fig. 10; while in Fig. 9 their position is below the mesoderm and lateral to the developing hind gut.

The positions of the most anterior sex-cells in embryo B′ 23 are indicated in Text Fig. *A*. The rectangle in this text figure includes the region which is drawn in detail in Fig. 8. Here a primary sex-cell is shown which is entirely free from any possible connection with the lateral mesoderm. It can scarcely be said to lie in, but rather lateral to the gut endoderm. It is half buried in the periblast. This fact suggests intimate relation with this nutritive layer. The cell figured is one of the few ever found with an irregular outline. This might seem to suggest amœboid activity, but this type is so extremely rare that it may be neglected from consideration.

Fig. 13 from B′ 23 shows a sex-cell which is .06 mm. to the rear of the one just mentioned. It is plainly in that portion of the lateral mesoderm which will develop into the splanchnic layer upon the formation of the cœlome (about the third day).

Observations of these early embryos show several important facts. The primary sex-cells are as truly characteristic and as easily recognizable as any found in the germ glands of later stages. They are located in the posterior half of the embryo, becoming gradually more numerous as the anterior part of this region is approached. Laterally they range from the extra-embryonic region to within the lateral mesoderm and the edge of the developing gut. In general their progress along the germinal path is directly proportional to the development of the embryo.

105-Hour Embryo.—Text Fig. *B* shows the relative positions of the sex-cells in the 105-hour embryo, B′ 26. As in previous cases the rectangle indicates the area from which Figs. 14, 15 and 16 were drawn. These three figures from the same embryo illustrate the full extent of the migration at this stage. On the left side of the embryo the sex-cells are found scattered all along the splanchnic mesoderm, from the region very near the split in the lateral mesoderm (Fig. 14) to that at the side of the gut (Fig. 16). On the right of Text Fig. *B* the sex-cells on the opposite side of the embryo are shown massed lateral to the gut. Should

the lateral mesoderm fuse above the gut, the formation of the dorsal mesentery would result and the position of the sex-cells would be identical to that found in later stages. Ex. Figs. 17 and 18.

6-Day Embryo. 2.6 Mm. Long.—Text Fig. *C* represents the position of the sex-cells as found in the 6-day embryo. At this time they are apparently in a state of rapid migration from the loose mesenchyme dorsal to the hind gut, to the positions ventral to the Wolffian ducts. Because of the laterally compressed condition of the embryo, which was due to the softness of the paraffin at the time of cutting, the transections are not exactly typical. However this embryo has been used since it represents most clearly the transitional stage between those figured in Text Fig *B* and *D*. Figs. 17 and 18 are detailed drawings of the 6-day stage. They illustrate the complete extent of the migration in the mesentery. The majority of the sex-cells were in the dorso-ventral position indicated by the cells in Fig. 17, while only a few were in that shown in Fig. 18. The more anterior sex-cells were farther along in the germinal path (being nearer the Wolffian ducts) than the more posterior ones. The position of the germ gland anlagen ventral to the Wolffian ducts is illustrated in Text Fig. *D*.

13-Day Embryo. 4 Mm. Long.—Text Fig. *E* represents the position of the germ gland anlagen as found in the 13-day embryo B 42 (4 mm.). The rectangle in Text Fig. *E* includes the region which is drawn in detail in Fig. 19. Rarely more than one sex-cell is found at this stage in any one section of a germ gland anlage. The anlagen are little more than protuberances from the peritoneum, containing relatively few peritoneal cells (although the sex-cells are surrounded by them) and they have not yet reached the future position of the gonads. It is obvious that the sex-cells which are contained in the peritoneal sac are all pushed ventrally by the developing swim bladder. One cell was observed in the position indicated by the cross in Text Fig. *E*. It was not included in the peritoneal sac and seemed apparently helpless in the loose mesenchyme ventral to the Wolffian duct. This cell had been delayed in reaching this position, had not been included in the sac, and in consequence of this fact it had not been

influenced by the action of the swim bladder. One of these lost cells is shown in the mesentery in Text Fig. *D*. The future of such cells is an open question.

A count of the sex-cells in this embryo, B 42, gave 64. There was never any question of recognizing these cells, for no cells of doubtful character were observed. Four of these cells found were in the mesentery above the gut, and one was in the loose tissue ventral to the Wolffian duct. No cases of disintegrating sex-cells were observed in our *Fundulus* material, although such conditions are reported by some investigators.

The *24-day embryo* shows the next advance in the germinal path. This stage has been considered perviously in connection with "Criteria of the Primary Sex-Cells."

Table of Average Diameters.

For the purposes of this investigation the diameters in micra were found by averaging the long and short dimensions of the cell and nucleus. By this method a number of representative sex-cells of embryos in all stages of migration were measured under the oil immersion and the following results were obtained.

Embryo.								Average.
1.B'21X (embryonic region) 46 hours	Cell	10.0	10.5	9.6	10.4			10.1
	Nucleus	6.0	6.4	5.6	5.2			5.8
2.B'21X (extra-embryonic) 46 hours	Cell	12.8	11.2	11.2	11.5			11.7
	Nucleus	6.0	6.1	6.5	6.5			6.3
3.B'26 105 hours	Cell	9.9	11.0	10.7	10.4	10.2		10.4
	Nucleus	6.1	6.2	6.2	6.1	5.8		6.1
4.B 30 6 days	Cell	12.3	12.4	12.7	11.2			12.2
	Nucleus	6.6	6.6	7.0	6.2			6.6
5.B 42 13 days	Cell	11.5	11.5	11.9	10.7	9.9	11.5	11.2
	Nucleus	7.2	6.6	8.0	7.8	5.7	6.3	6.9
6.B 65 24 days	Cell	11.5	11.8	10.2	10.4	9.0	9.5	10.4
	Nucleus	6.1	7.8	7.3	7.0	6.2	6.6	7.2

Multiplication of the Sex-cells.

The early distribution of the sex-cells (Figs. 20 and 21) is best explained, we believe, in connection with the streaming of the organ-forming substances which contribute materials to the embryo body. In most processes of this nature not only cell trans-

portation but cell division takes part. Certain workers with other forms have held that the movement of the cells into the anlagen of the gonads is not the only factor responsible for their increase, but that multiplication actually occurs during the period of translocation. Mitotic figures have never been observed in *Fundulus* among the recognizable sex-cells which are within the embryo, although a most thorough search has been made for them in many embryos at all stages of development. A count of these cells in several specimens in various stages reveals the fact that there is a tendency for their number to vary more or less from the average established (67). However there is not enough variation to convince one that there is any marked multiplication of the sex-cells during the migration period. These facts naturally lead to the conclusion that the first period of multiplication takes place in the extra-embryonic region.

In the description of the earliest embryos referred to in this report and in the figures presented, emphasis has been placed upon the fact that the primary sex-cells in any one embryo are not in the same phase of migration. Furthermore observations upon all stages show that development becomes more advanced anteriorly than posteriorly. It is of further interest that in embryos containing sex-cells both within and without the body, the number falls below the average for older stages. These conditions and the fact that no sex-cells have been found in any region other than that already described, suggest the explanation that these cells multiply in the extra-embryonic region. Indeed the four sex-cells illustrated in Fig. 12 may indicate recent cell division by their very association. If they are not of recent and identical origin they would probably be farther separated than they are in this figure. These views are presented only tentatively, due to lack of sufficient material to warrant definite statements on this multiplication; for no mitotic figures have ever been seen in the extra-embryonic region to substantiate this belief in a division as suggested. Our material has not permitted a careful study of this matter. However considering the longitudinal distribution of the sex-cells in the earliest available embryos, and their tendency to approach a common average number in each individual, one is inclined to regard them as being of unquestionably

earlier origin than it has been possible thus far to trace them. It seems not unreasonable to believe that the fore-runners of these cells have been segregated at a time very early in the development of the germ ring.

Discussion and Conclusions.

This paper attempts to identify definitely the sex-cells which are present in the 24-day embryo as the "primordial germ cells" of previous writers, or as the "primary genital cells" of Firket. It also presents evidence on the manner in which these cells reach their final destination.

The method of embryo formation in the teleosts has a bearing upon the question of sex-cell migration in *Fundulus*. It will be recalled that the anterior portion of the embryo is formed from the head fold, which may perhaps be nothing more than a thickening on the germ ring; while the body or posterior portion is to be regarded as the result of the developmental process termed concrescence. It is only this latter portion of the body that is involved in the formation of the sex-cells. The eggs have a large amount of yolk, and a very distinct germ ring. As cell proliferation takes place, the germ ring moves gradually downward over the yoke mass. The primitive streak moves backward and receives the converging limbs of the germ ring posteriorly. The material of the halves of the germ ring, after fusion, is differentiated into the embryo posterior to the head process. The rudiments of the embryo body are not clearly marked out in *Fundulus* until the germ ring is completely closed.

The earliest primary sex-cells which we have located are from embryos in which the germ ring has been closed but a few hours, and in which the tail is just beginning to elongate. Their position in the extra-embryonic region lateral to the undifferentiated endodermal cell mass at the posterior half of the embryo is indicated in Fig. 20. In other embryos of the same stage of development, numerous primary sex-cells are present in practically the identical relation to the embryo that is clearly demonstrated in Fig. 20. These sex-cells invariably lie just above the periblast and are associated with the sheet of cells which is a lateral expansion of the undifferentiated endodermal cell mass

(peripheral endoderm, Allen). The complete germinal path from this position to one lateral to the hind gut may be followed in almost any embryo of from 46 to 50 hours. This very advantageous condition is made possible by the greater development near the middle of the embryo, for it is only a natural result of embryo formation by concrescence that development is progressively greater anteriorly from the point of convergence of the germ ring.

These cells are transported from the edge of the embryonic region medially, to positions just beneath or within the endodermal cell mass, as the case may be. They are carried passively from one position to another by the same forces of growth which bring together the halves of the germ ring. The influence of this factor can scarcely be over emphasized. Although not outwardly as apparent as in earlier stages, these forces are nevertheless responsible for the flowing of the streams of embryonic material towards the future position of the organs which are to develop therefrom.

The sex-cells come to lie within these shifting layers of embryonic endoderm and mesoderm and naturally accompany these layers in their changes of position. Because of the fact that the movement of the sex-cells is not active but rather dependent upon that of surrounding layers, the expression "migration" seems rather unfortunate. Some term such as "translocation" would perhaps be more truly expressive of the actual conditions.

These cells come to lie in the edge of the embryonic region, and when a portion of the undifferentiated cell mass gives rise to gut endoderm and another to lateral mesoderm, they follow one layer or the other. The sex-cells follow one or the other of these layers until they reach a position lateral to the newly formed gut. Which layer is chosen apparently depends upon chance. Those cells which have been carried in the edge of the endoderm never enter the gut, but move dorsally from the side of it into the lateral mesoderm. Here they join the sex-cells which have been carried in the mesoderm. By this time the split, resulting in the formation of the cœlome between the splanchnic and somatic mesoderm has taken place. Although the sex-cells are associated with all parts of the lateral mesoderm before the forma-

tion of the cœlome, it is a noteworthy fact that they never occur within the somatic layer after differentiation.

From this position lateral to the hind gut the cells are in the general dorsal movement of the mesoderm which eventually results in the formation of the intestinal mesentery. The cells from either half of the embryo remain apart and seem to lie in separate streams of mesodermal cells which are flowing toward the Wolffian ducts. But although there may be a pause here, at no time do the sex-cells appear to establish any intimate relation with the cells of these ducts (Text Fig. *D*). From the evidence at hand, an explanation of the function of these cells which makes them contributory to the development of the already well-formed Wolffian ducts, as suggested by certain investigators, does not seem plausible in *Fundulus*.

As the sex-cells reach a position nearly ventral to the Wolffian ducts they become surrounded by a single layer of peritoneal cells. This covering develops until the position of the future sex organs is attained; the sex-cells then rest in sac-like protuberances from the peritoneum, the germ gland anlagen. Assisting in the movement which brings the sex-cells into their future positions, are several factors entirely external to the germ glands. For example, there is a rapid proliferation of the loose mesenchyme dorsal to the gut and the development of the swim bladder which results in a median down pushing. The ventral movement (Text Figs. *D* and *E*) from the region of the Wolffian ducts is clearly due to the wedge-like effect produced by the growing swim bladder. That this process is necessarily passive is evident from the fact that amœboid activity of the cells included within the germ gland would be unable to produce any change in its position.

From the evidence in *Fundulus* it is apparent that the sex-cells enter the embryo and are located in the germ glands by the same forces that are influential in the distribution of the other organ forming substances of the body. Their "migration" is not to be looked upon as different from that of any other group of cells. But while the sex-cells are not amœboid, there is nevertheless reason for misunderstandings which have arisen regarding their activities. In the first place they are relatively few in compari-

son to the great numbers of cells in the surrounding tissues. Although the entire mass of cells is continuously in motion, only the movement of the sex-cells is at all noticeable. They are shifted about by the active surrounding tissues and naturally assume slightly irregular outlines at times, due to the unequal tension upon the cell membrane. Through a misinterpretation of the conditions within the embryo these sex-cells may easily be accredited with peculiar powers of locomotion. A sex-cell, as a slowly drifting cloud, can be seen gradually to change its position; but movements of the tissue cells about it, due to their location in continuous layers, are so inconspicuous as to go unnoticed. Because of this, the movement of the sex-cells should be considered merely as the passive indication of the rate and direction of progress of contiguous layers.

SUMMARY.

1. The earliest primary sex-cells found in *Fundulus* were located in the peripheral endoderm, lateral to the posterior half of the 46-hour embryo. No sex-cells were observed in that part of the embryo which develops from the head fold.

2. The germinal path leads from the peripheral endoderm, into the border of the undifferentiated endodermal cell mass. When this cell mass splits to form gut endoderm and lateral mesoderm, the sex-cells proceed medially with either layer. By the time the gut is formed, these cells are lateral to it; they all eventually become located in the splanchnic mesoderm of this region. From here the sex-cells migrate dorsal to the hind gut, thence to the region ventral to the Wolffian ducts. Here they become surrounded by peritoneal cells which form the somatic portion of the gonads. From this position the germ gland anlagen are shifted back to their final location dorsal to the gut.

3. There is very little multiplication of the sex-cells during the period of migration. Division apparently takes place in the extra-embryonic area, and is not renewed to any marked extent until after the sex-cells become located in the germ glands.

4. The constant distinguishing characteristics insure positive identification of these cells throughout all phases of their migration, and leave no reason to question their identity as being the "primordial germ cells" of previous writers.

5. Migration is passive, being due to forces of growth which are altogether external to the cells themselves. These forces of growth are factors common to the development of the organs formed in the body of the teleost embryo.

6. Evidence derived from this study of *Fundulus* is in absolute harmony with the theory of early segregation of these primary sex-cells.

Note

Some time after the manuscript of this paper had been sent to the press, an extensive article by Okkelberg, entitled, "The Early History of the Germ Cells in the Brook Lamprey, *Entosphenus wilderi* (Gage), up to and Including the Period of Sex Differentiation," appeared (*Jour. Morph.*, Vol. 35, No. 1, 1921). This article contains much data and many important conclusions, and it is to be noted (on pages 35 and 36) that the author, in considering the sex cells, has discussed their methods of migration. It is of great interest that the conclusions reached by Okkelberg on this matter for the lamprey are very similar to our own upon *Fundulus*.

BIBLIOGRAPHY.

Allen, B. M.

'04 The Embryonic Development of the Ovary and Testis of the Mammalia. Amer. Jour. of Anat., Vol. 3.

'11 The Origin of the Sex-Cells of *Amia* and *Lepidosteus*. Jour. of Morphology, Vol. 22, No. 1.

Dodds, G. S.

'10 Segregation of the Germ-Cells of the Teleost *Lophius*. Jour. of Morphology, Vol. 21.

Eigenmann, C.

'92 On the Precocious Segregation of the Sex-Cells of *Cymatogaster*. Jour. of Morphology, Vol. 5.

'96 Sex Differentiation in the Viviparous Teleost *Cymatogaster*. Arch. für Entw. Mech., Bd. 4.

'02 Viviparous Fishes of the Pacific Coast (*Cymatogaster aggregatus*). Bull. U. S. Fish Comm., Vol. XII, p. 412.

Firket, F.

'20 On the Origin of the Germ-Cells in the Higher Vertebrates. Anat. Record, Vol. 18, No. 3.

Jarvis, May.

'08 The Segregation of the Germ Cells of *Phrynosoma cornatum*. Preliminary Note. Biol. Bulletin, Vol. 15, No. 3.

Jordan, H. E.

'17 Embryonic History of the Germ-Cells of the Loggerhead Turtle. Pub. No. 251 Carnegie Inst. of Wash., pages 313 to 344.

King, H. D.

'08 The Oögenesis of *Bufo Lentiginosus*. Jour. of Morphology, Vol. 19 p. 369.

Nussbaum, M.

'80 Zur Differenzierung des Geschlechts im Tierreich. Arch. f. mikr. Anat., Bd. 18.

Swift, C. H.

'14 Origin and Early History of the Primordial Germ-Cells in the Chick. Amer. Jour. of Anatomy, Vol. 15, No. 4.

'15 Origin of the Definitive Sex-Cells in the Female Chick and their Relation to the Primordial Germ-Cells. Amer. Jour. Anat., Vol. 18, p. 441.

'16 Origin of the Sex-Cords and Definitive Spermatogonia in the Male Chick. Amer. Jour. Anatomy, Vol. 20, p. 375.

Wilson, H. V.

'89 Embryology of the Sea Bass. Bull. U. S. Fish Comm., Vol. 9.

Woods, F. A.

'02 Origin and Migration of the Germ-Cells in Acanthias. Amer. Jour Anat., Vol. 1, No. 3.

EXPLANATION OF ILLUSTRATIONS.

All figures in this report were drawn with the aid of a camera lucida. Any lens combinations which were considered necessary to produce the best results were used. The magnification as given for each figure was calculated carefully and is correct for the reproductions as they appear on these plates.

ABBREVIATIONS.

a., anus.
Ao., aorta.
B., blood cells.
c., centrosome.
cr., chromatin knots.
Co., cœlome.
Ect., ectoderm.
E.M., endodermal cell mass.
En., gut endoderm.
G., germ gland anlagen.
g., gut.
L.M., lateral mesoderm.
li., linin network.
M., mesoderm.
Mes., gut mesentery.
N, notochord.
Nt., neural tube.
nu., nucleolus.
P., peritoneum.
pe., periblast.
P.N., periblast nucleus.
S., sex-cell.
sw., swim bladder.
So., somatic mesoderm.
Sp., splanchnic mesoderm
T., elongating tail.
Wo., Wolffian duct.

DESCRIPTION OF ILLUSTRATIONS.

PLATE I.

FIG. 1. A typical primary sex-cell from a 105-hour embryo (B′ 26). Showing the large centrosome, two nucleoli and chromatin knots scattered over the periphery of the nucleus. × 1420.

FIG. 2. A typical sex-cell from a 6-day embryo (B 30). Chromatin granules finer than in the preceding cell. × 1420.

FIG. 3. Sex-cell from a 100-hour embryo (B′ 25–3). From the extra-embryonic region. It is closely associated with the periblast. In other sections the peripheral endoderm may be seen out over this area. × 1420.

FIG. 3*a*. Sex-cell from a 9-day embryo (B 34). Chromatin arrangement is intermediate between that found in Figs. 1 and 2. × 1420.

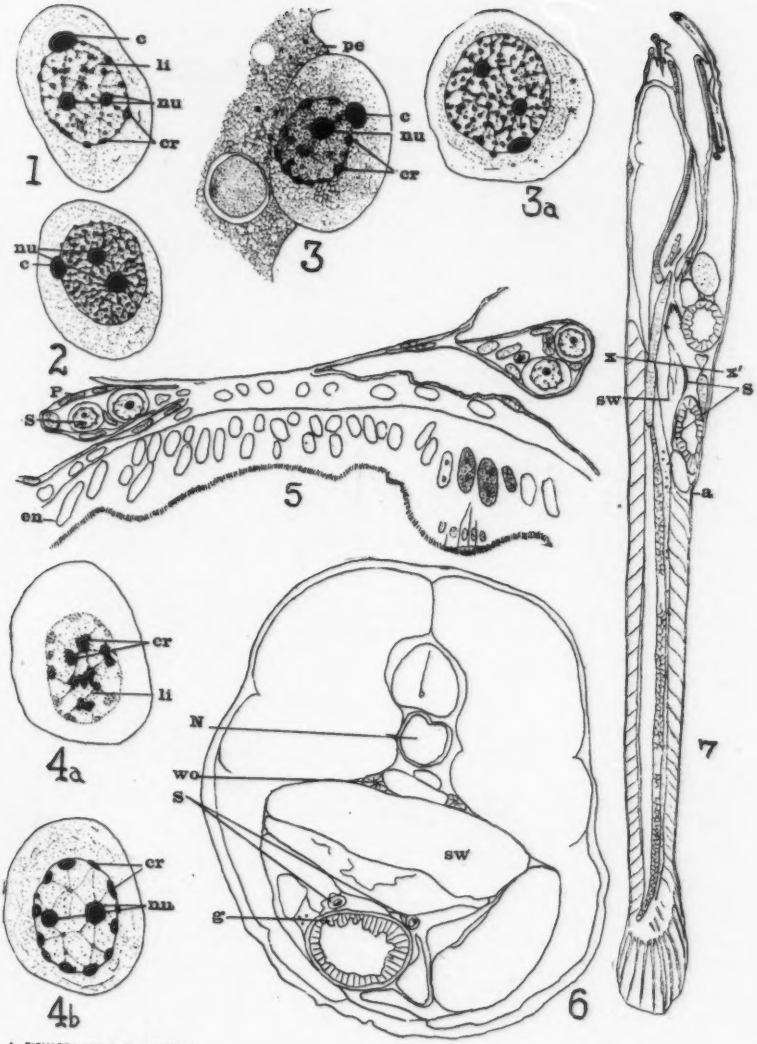
FIG. 4*a*. Surface view of a nucleus from a sex-cell in a 105-hour embryo, showing the chromatin knots in surface view. × 1420.

FIG. 4*b*. From the same nucleus as the one used in Fig. 4*a*. Obtained by focusing upon the center of the nucleus, illustrating the chromatin knots in optical section. × 1420.

FIG. 5. Showing the sex-cells in the germ gland anlagen dorsal to the hind gut of a 24-day embryo. The function of the peritoneal cells at this stage is quite evident in this illustration. The sex-cells are completely surrounded by mesoderm. × 700.

FIG. 6. Transection through a 24-day embryo (B 65–2) taken at the position indicated by the plane X–X′ in Fig. 7. Showing the position of the gonads in relation to other parts of the embryo; especially as regards the developing swim bladder. × 120.

FIG. 7. Longitudinal section lateral to the median line of the 24-day embryo (B 65). Illustrating the position of the gonads as being the same as in the adult. Migration ceases at this point. The activities within the gonads after they have reached this point of development will not be considered at this time. × 25.



A. RICHARDS AND J. T. THOMPSON

PLATE II.

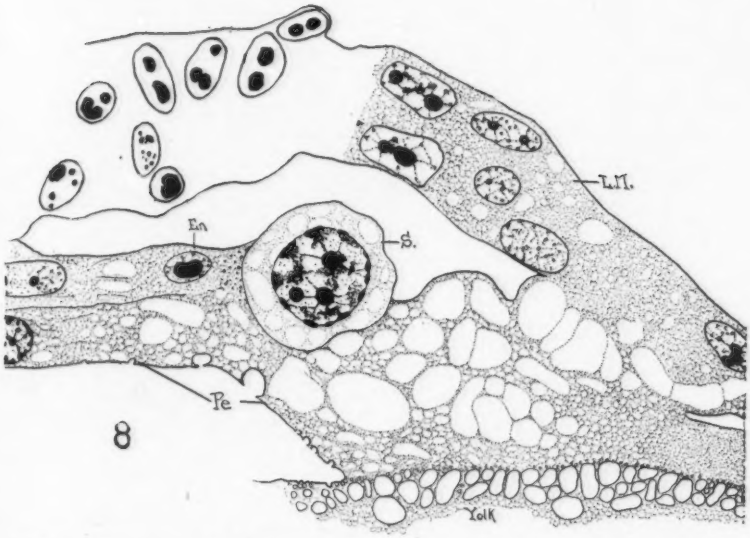
FIG. 8. A sex-cell from the 50-hour embryo (B′ 23). Showing in detail the region at the edge of the embryo, where the germ layers meet the periblast. The sex-cell is in the edge of the gut endoderm, entirely removed from any connection with the lateral mesoderm, and is partially imbedded in the periblast. Several very early cells have been found in this relation to the periblast, but so far it has been impossible to establish any significance to this fact. Due to exertion of unequal tension upon the cell membrane, the cell outline appears slightly irregular. × 1400.

FIG. 9. Semi-diagrammatic transection of the 46-hour embryo B′ 21 X taken at the position indicated by the line "*C*" in Fig. 20. At this early stage the lumen of the gut is not formed completely, even in this most anterior region. The sex-cells lie between the periblast and the lateral mesoderm, at the side of the developing hind gut. × 300. (The positions of the sex-cells in the germinal path correspond to certain stages in development of the gut.)

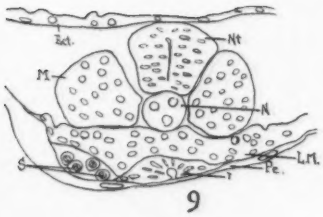
FIG. 10. Transection of embryo B′ 21 X taken at the position indicated by the line *B* in Fig. 20. Here the gut endoderm and the lateral mesoderm are differentiated to a certain extent, although the splitting of the endodermal cell mass has not yet occurred. The sex-cell lies above the periblast in the edge of the cell mass. × 300.

FIG. 11. Transection of embryo B′ 21 X taken at the line "*A*" in Fig. 20. The sex-cell illustrated is in the extra-embryonic region, associated closely with the peripheral endoderm. × 300.

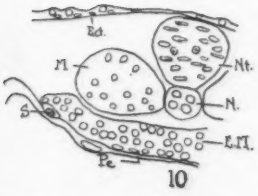
FIG. 12. A longitudinal section through the elongating tail of the 46-hour embryo B′ 21 L. The 4 sex-cells illustrated are in the peripheral endoderm at the extreme posterior of the embryonic area. × 300.



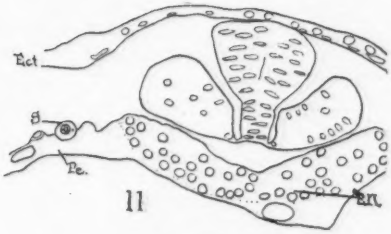
8



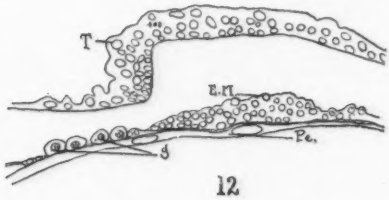
9



10



11



12

Plate III.

Fig. 13. Sex-cell from the 50-hour embryo B′ 23. It lies in the extreme edge of the lateral mesoderm, just dorsal to its separation from the periblast. This cell lies in that portion of the mesoderm which will give rise to the splanchnic layer. × 700.

Fig. 14. A sex-cell in the splanchnic mesoderm of the 105-hour embryo B′ 26. The cell is just medial to the point of differentiation between the somatic and splanchnic layers. This stage also shows an advance over the one illustrated in Fig. 13, in that the gut and Wolffian duct have taken form. × 700.

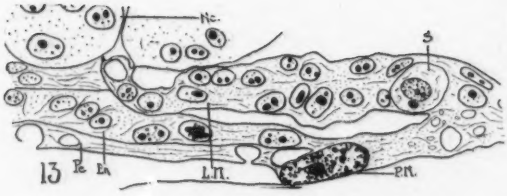
Fig. 15. A group of sex-cells from the region anterior to that drawn in Fig. 14. Here the sex-cells are approaching the side of the hind gut. × 700.

Fig. 16. A sex-cell from embryo B′ 26, in the region anterior to those illustrated in Figs. 14 and 15. The more medially placed cell is in the splanchnic mesoderm lateral to the gut. As this layer grows up over the gut to form the dorsal mesentery, the sex-cells will naturally be brought to lie in this region. × 700.

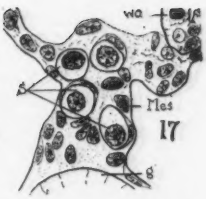
Fig. 17. Four sex-cells from the 6-day embryo B 30 in the mesentery dorsal to the gut. This shows in detail the region included in the rectangle in Text Fig. *C*. The extraordinary width of the mesentery at this stage is doubtless due in part to the presence of the sex-cells. × 420.

Fig. 18. This figure illustrates the extremes of the migration at this stage. No cell was found at any earlier stage than the one near the gut, none later than that ventral to the Wolffian duct. From embryo B 30. × 420.

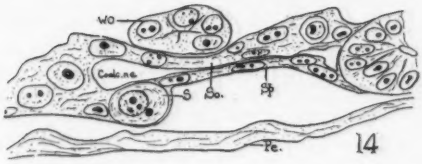
Fig. 19. Showing the development of the gonads in the 13-day embryo B 42. The sex-cells are fixed in sac-like protuberances from the peritoneum. From this figure it is possible to obtain an idea of the effect produced by the rapid growth of the swim bladder, in literally pushing the gut and all related tissues ventrally. It is also interesting to observe that the peritoneal covering renders this cell dependent upon surrounding tissues for movement to its final position dorsal to the gut. × 420.



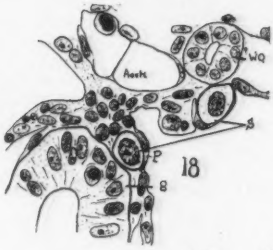
13



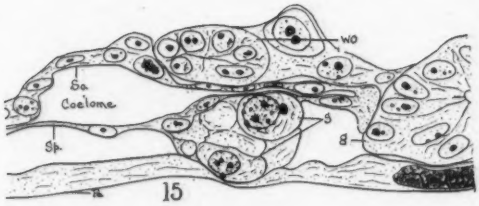
17



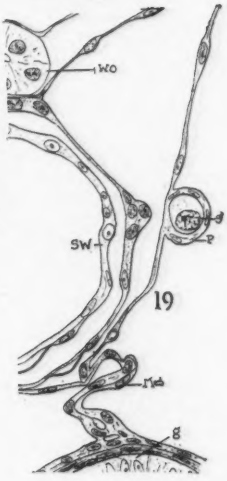
14



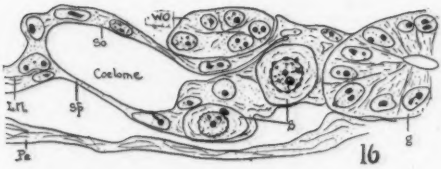
18



15



19

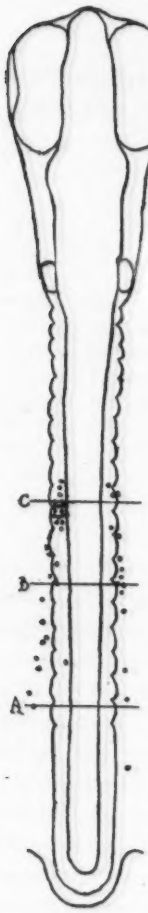


16

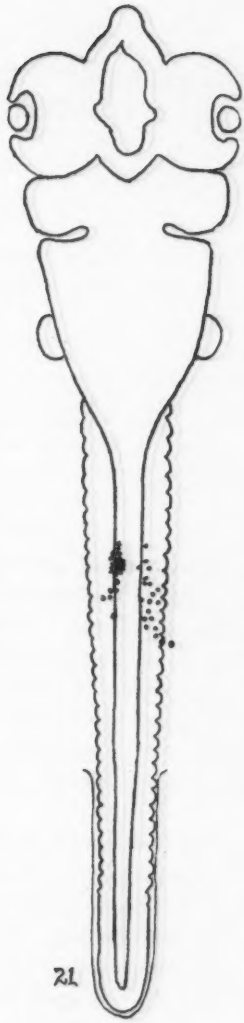
PLATE IV.

FIG. 20. A diagrammatic reproduction of embryo B′ 21 (46 hours) demonstrating the distribution of the sex-cells at this early stage. The variation in anterior-posterior development is very noticeable and is explained fully in the text. × 65.

FIG. 21. Reproduction of embryo B′ 26 (105 hours) constructed similarly to Fig. 20. Showing the positions of the sex-cells as being more medially placed than in the earlier stage. The cells on the right are not as far along with migration as those on the left; the former group migrated with the mesoderm and the latter followed the gut endoderm. × 65.



20



21

A. RICHARDS AND J. T. THOMPSON.

SPERMATOGENESIS OF APHIDS; THE FATE OF THE SMALLER SECONDARY SPERMATOCYTE.

H. HONDA.

CONTENTS.

I. Introduction 349
II. Method 350
III. *Stomaphis yanois* 350
1. Primary Spermatocyte 350
2. Larger Secondary Spermatocyte 351
3. Smaller Secondary Spermatocyte 352
4. Smaller spermatid 353
IV. *Neothomasia populicola* and *Macrosiphum ambrosiæ* 356
V. Review 358
VI. Summary 360

I. INTRODUCTION.

It has been shown by Morgan and von Baehr that in aphids the primary spermatocyte divides unequally producing larger and smaller secondary spermatocytes. The larger secondary spermatocyte undergoes a second maturation division, and produces two equal-sized spermatids which transform into functional spermatozoa. The smaller secondary spermatocyte, which has received fewer chromosomes is said to degenerate. Only two similar spermatozoa, consequently, are formed from a primary spermatocyte.

Von Baehr (1909 and 1912) states that he very rarely observed the development of the smaller secondary spermatocyte to the prophase of the second maturation division, but that it does not divide. Stevens (1909) says that in a preparation which has unfortunately been lost, the anaphase of the smaller secondary spermatocyte was seen, and that such stage may also be distinguished among the degenerating spermatocytes. Morgan (1915) states: "The small cell is left with two chromosomes and a small amount of cytoplasm. It never divides again, and later degenerates. Stevens was inclined to think that the small cell may

sometimes show a division figure, which subsequently fades away, but I have never seen a case of this kind."

In *Macrosiphum ambrosiæ* and *Neothomasia populicola* I have observed the late telophase of the smaller secondary spermatocytes. In *Stomaphis yanois*, moreover, I have found that the smaller secondary spermatocytes divide and form equal-sized spermatids which are much smaller than the larger spermatids. These smaller spermatids develop and reach the sustentacular cells with the developed larger spermatids; they, however, fail to attach to the sustentacular cells. Thus their development ceases; they, therefore, do not fully transform into spermatozoa, but retrogress and form spherical cells, which attach themselves to the epithelium of the cysts of the testes. A further account of this will appear in the following pages.

The work on the spermatogenesis of *Stomaphis yanois* was done in the Tokyo Higher Normal College, and the work on the other aphids has been done in the University of Chciago. The writer's thanks are due to Prof. F. R. Lillie and Prof. S. Yamanouchi, who gave him many suggestions and much help. The writer also wishes to thank Prof. A. Oka and Prof. U. Takakura for their kindness during his stay in Tokyo. For the identification of the aphids the writer is indebted to Dr. A. L. Quaintance and Dr. A. C. Baker.

II. Methods.

Both the males and parthenogenetic females were fixed in either strong Flemming's, Zenker's or a mixture of absolute alcohol one part, acetic acid one part, and saturated aqueous solution of corrosive sublimate two parts. Sections were cut 3, 5 and 10 micra in thickness; most of them, however, were cut 5 micra thick. They were stained with Heidenhain's iron-hematoxylin followed by eosin or borax carmin.

III. Stomaphis yanois.

1. *Primary Spermatocyte.*

Figs. 1 and 2 show the primary spermatocyte prophase. In Fig. 1, one of the chromosomes is formed, and in Fig. 2, the proc-

ess of the formation of the chromosomes is almost finished. There are ten chromosomes, five larger and five smaller, in the equatorial plate of the first spermatocyte division, and they are connected with one another by linin threads as is shown in Figs. 4 and 5. The side view of the mitotic figure shows centrosomes of about the same size agreeing with von Baehr's observation on *Aphis saliceti* (Fig. 3).

In the anaphase unequal cell division is indicated. The larger and smaller daughter cells are connected by a bridge of cytoplasm, and elongated lagging chromosomes lie between the chromosomes passing to the daughter cells (Fig. 7). The lagging chromosomes do not show any tendency to go to the larger cell at this time, but after the nuclear membrane is formed, the lagging chromosomes enter the larger cell (Fig. 8). It is interesting to note that the size of the nuclear membrane is larger in the larger cell. The inequality of the size of the nuclei of the daughter cells, therefore, does not seem to be due to the unequal number of the chromosomes, but to an unequal quantity of cytoplasm. In a case where the two daughter cells were about equal the size of the nuclear membrane was about the same.

I have observed many cases in which the lagging chromosomes appear to be divided, but I doubt that this ever occurs. Morgan (1909) states: "That artificial conditions, such as handling or osmosis, might break such a delicate connection at this time is not at all improbable, and such an artificial result might give the impression that the accessory is actually divided. Moreover, if the bridge arches toward or away from the observer, the effect may be produced at certain focal levels of discontinuity between the ends of the lagging chromosomes, when none such exist."

The larger secondary spermatocyte receives eight divided and two lagging undivided chromosomes, and the smaller secondary spermatocyte receives eight divided chromosomes.

2. *Larger Secondary Spermatocyte.*

The larger secondary spermatocyte undergoes an equal second division without an intervening resting stage. The equatorial plate (Figs. 12 and 13) of the second division shows ten chro-

mosomes. In the first maturation division five of the ten chromosomes are larger and five of them are smaller, but in this case six are larger and four are smaller. The reason for this is discussed later on. As in the case of the first division, chromosomes are connected by linin threads. When the split chromosomes shift to the opposite poles interzonal fibers appear. In the first division the middle part of the two daughter cells becomes narrow and shows an appearance of a dumb-bell with the ends different in size. In this case, however, the middle part is broad, so that the interzonal fibers are separated (Fig. 15).

3. *Smaller Secondary Spermatocyte.*

The smaller secondary spermatocyte shows chromosomes in its nuclear cavity at the telophase of the first maturation division. It is not difficult to distinguish the smaller secondary spermatocyte as their diameter is hardly half that of the larger ones. The nucleus does not enter a resting stage. I have found in some cases two small bodies near the nuclear membrane (Fig. 9). These seem to be centrosomes, but I am unable to speak with certainty. The changes in preparation for the second division are similar to those of the larger spermatocyte.

The equatorial plate (Figs. 20 and 21) shows eight chromosomes as compared with ten chromosomes in the equatorial plate of the larger secondary spermatocyte. The cases which distinctly show eight chromosomes are rare; there can be little doubt, however, that this is the full number since there are ten chromosomes in the equatorial plate of the first maturation division, and two of them pass to the larger one as the lagging chromosomes. Four chromosomes are larger and the other four are smaller. There are five larger and five smaller chromosomes in the equatorial plate of the first division; the lagging chromosomes, therefore, must be a larger and a smaller chromosome. If all the chromosomes were to divide in the first division, five larger and five smaller chromosomes would appear in the equatorial plate of the second division. Two chromosomes, one larger and one smaller, lag and enter the larger cell without dividing. The smaller of the lagging chromosomes, consequently, becomes larger than the

other smaller chromosomes. This must be the reason why we see four smaller chromosomes in the larger secondary spermatocyte instead of five.

The side view of the metaphase of the smaller spermatocyte differs from that of the larger one in shape. It is more spindle-shaped (Fig. 22). Fibers are not seen distinctly in the preparations stained with iron hematoxylin. The two stained bodies on both sides of the chromosomes in the equatorial plate might be the centrosomes (Fig. 23). There are cases which show separated chromosomes, and cases which show massed chromosomes (Figs. 23–25). So far as my observation goes, in most cases the chromosomes seem to fuse soon after their splitting. The telophase does not show distinctly the interzonal fibers as in the case of the larger cell. Two equal smaller spermatids are produced after the division.

4. *Smaller Spermatid.*

The germ cells of each cyst of the testes are generally in about the same stage. When the spermatids are young the cysts are spherical in shape, but they elongate during the development of the spermatids. The young smaller spermatids (Fig. 28) have condensed nuclei, but the larger spermatids (Fig. 18) between which they lie have vesicular nuclei. These smaller and larger spermatids are seen all through the cyst. I have examined many cases in order to see whether the polarities of the larger and smaller spermatids are established with relation to the epithelium or not. Most of the young larger and smaller spermatids, which are seen near the epithelium, develop their tails toward the center of the cyst, but some of them may develop along the epithelium or develop their tails toward the epithelium. Those in the central part do not show any definite orientation, and in extreme cases spermatids existing side by side may show opposite directions. In cysts in which the larger spermatids are developed to the stage shown in Fig. 18, the orientation of the larger and smaller spermatids remains unchanged. In a little later stage, however, all the larger and smaller spermatids begin to orient in the same direction, and when the larger spermatids develop to the stage shown in Fig. 19, all are oriented in the same direction.

There must be an interaction, probably chemical, between the sustentacular cells and the larger and smaller spermatids. The larger and smaller spermatids in the outer part opposite the sustentacular cells and in the central part of the cyst generally move among the tails of the other spermatids toward the sustentacular cells, but those in the other parts move toward sustentacular cells along the epithelium.

Developed smaller spermatids (Fig. 31) are seen among the larger spermatids near the sustentacular cells, and do not show any inferiority to the larger spermatids in moving toward the cells. Before the nucleus of the larger spermatid shows marked differentiation the smaller spermatids have retreated a little towards the interior. In other words, well developed smaller spermatids approach towards the sustentacular cells, but do not attach to them. I have examined many smaller spermatids in order to see whether they develop apical parts. Figure 30 shows a developing smaller spermatid, which has a cone-shaped apical part. There is a developed smaller spermatid,* which seems to have a well-developed apical part, but we cannot distinctly observe since it is seen in close contact with the tails of the larger spermatids. In most of the smaller spermatids, which have elongated tails, I have not, however, observed developed apical parts.

As to the interpretation of the cells identified as smaller spermatids, may they not be degenerating larger spermatids? So far as my observation goes the larger spermatids rarely degenerate; moreover, it is not hard to distinguish degenerating young larger spermatids from the smaller spermatids, since the former are not only much larger than the latter, but the nucleus of the larger spermatid becomes vesicular while the nucleus of the smaller spermatid is condensed. If the larger spermatids developed to the stage shown in Fig. 19 begin to degenerate, we can recognize them by the difference in the state of the nuclei. If the almost fully developed spermatids begin to degenerate, it is quite easy to tell them from the smaller spermatids, since they have very slender nuclei and the smaller spermatids, which are seen in the same cyst with them, have spherical nuclei. If degeneration of the larger spermatids should occur at the stage in which they

have condensed ovoid nuclei which elongate later, the criterion by which to distinguish them is their position, since when they have developed to such a stage, the smaller spermatids with condensed spherical nuclei have already left the epithelium.

The metaphase of the smaller secondary spermatocytes are seen among those of the larger secondary ones; I think, therefore, there is no doubt that the smaller secondary spermatocytes undergo the second division. More developed larger spermatids are seen with more developed smaller spermatids in the same cyst. We may conclude from these observations that the smaller spermatids develop with the larger spermatids.

I have observed cases where the larger and smaller spermatids are seen in the central part of the cyst, while the majority of spermatids have already reached the sustentacular cells. Such larger and smaller spermatids might fail to reach the sustentacular cells, since they have to move among the spermatids. The examination of the later stages, however, has shown that they succeed in reaching the sustentacular cells.

Figure 32 shows a smaller spermatid which is abnormally big and has a distinct axial filament. Ordinarily the smaller spermatids elongate similarly, but are more delicate. One of the most developed smaller spermatids is shown in Fig. 33. In such a stage their development comes to a standstill, and they begin to retrogress. They gradually retreat toward the tails of the larger spermatids. Their nuclei which are deeply stained with iron hematoxylin are seen among the tails of the larger spermatids in a somewhat regular position. Finally they pass out to the cavity of the cyst.

The smaller spermatids fail in attaching to the sustentacular cells; they cannot, consequently, get material for their further development. They have to live on their own substance. Their tails become shorter, and the cytoplasm around the nucleus increases (Fig. 34).

The forms shown in Fig. 35 are seen near the tail of the fully developed functional spermatozoa in the cavity of the cyst. We do not see such spermatids in the cavities of the cysts at the younger stages. These smaller spermatids still have elongated tails, but later transform into spherical cells which have a distinct cell

membrane (Fig. 39) and show a tendency to fuse with each other. There are some cells which have two or more condensed nuclei. These seem to be the products of the fusion of two or more smaller spermatids. Some of the retrogressed cells of the smaller spermatids attach to the epithelium, and on these cells other cells attach themselves; thus they form layers as shown in Fig. 38. In other cases they are irregularly attached to the epithelium. When they attach themselves to each other they show a polygonal shape.

A, *b* and *c* in Fig. 38 are parts of adjacent cysts, where fully developed spermatozoa occur though not shown in the figure. The cells occurring between the cysts are the retrogressed smaller spermatids produced in the cyst *c*, and the epithelium proper is very thin as seen between cysts *a* and *b*. As we see in the figure these cells are not equal in size. In some of them the nuclei are broken up and their fragments are seen scattered throughout the cells. The others still show condensed spherical nuclei. As stated already the larger spermatids rarely degenerate. These larger spermatids may become like the cells just mentioned. Though degenerating larger spermatids mingle among these cells, there is no criterion by which they may be distinguished from retrogressed cells of the smaller spermatids.

Some of these cells may be absorbed by the epithelial cells, but how far the absorption proceeds is at present undetermined. When these cells attach to the epithelium the functional spermatozoa are already fully developed. Afterwards the wall of the cyst ruptures, and these cells being deprived of their connection with the testis are destined to disappear. It is possible that they are extruded from the testis along with the spermatozoa. I have observed epithelial cells of the cyst and retrogressed cells of the smaller spermatids in some of the vasa deferentia. The sections of the testes of the old males show remarkable changes. Their walls are thickened and neither spermatozoa nor the cysts, which fill the young testes, can be seen.

IV. Neothomasia populicola and Macrosiphum ambrosiæ.

The testes of embryos of *Neothomasia populicola* and *Macrosiphum ambrosia* are in the early stages, but those of larvæ are

suitable for the purpose of studying the spermatocyte divisions. As is the case in other aphids, the primary spermatocytes of these aphids divide unequally, and the anaphase shows the lagging chromosomes. I have found in these aphids telophases of the second maturation division of the smaller secondary spermatocyte, but have observed no developing smaller spermatid; we may, therefore, conclude that the smaller secondary spermatocytes and the smaller spermatids of these aphids degenerate as in the cases of the aphids studied by Morgan, von Baehr and Stevens. In *Macrosiphum ambrosia* I observed cases in which all smaller secondary spermatocytes seemed to be dividing, but I will conclude in a succeeding paper whether all the smaller secondary spermatocytes divide or not.

In the cyst, where larger spermatids are already attached to the sustentacular cells, there are seen spermatids which look like the smaller spermatids of *Stomaphis yanois*. As stated above, since no development of the smaller spermatids was observed, they must be larger spermatids. In slightly younger cysts some spermatids are seen among the developed tails of other larger spermatids, which are about to attach to the sustentacular cells. Such spermatids probably have no chance of reaching the cells. I have found cases in which the larger spermatids are already attached to the cells, but some spermatids are seen among the ends of the tails of the larger spermatids. In other cysts spermatids with condensed nuclei are seen apart from the sustentacular cells, while others are attached to them.

As in the case of *Stomaphis yanois* young spermatids of these aphids change their orientation to the same direction; some spermatids, therefore, move to the sustentacular cells across the whole diameter of the cyst or reach the cells moving along the epithelium. If they move to the sustentacular cells along the epithelium, as most of the spermatids do, they may lose the chance to become attached to them. Developed spermatids have been found by the side of spermatids which are attached to the sustentacular cells and are developing. They were probably prevented from reaching the cells by other spermatids, and their development came to a standstill; they, consequently, show younger stages than the spermatids which are attached to the

cells. Since many spermatids are produced in the cysts, if they move to the sustentacular cells through the tails of other spermatids, they meet much resistance; they, therefore, might be unable to reach the cells.

The most conspicuous difference between the case of *Stomaphis yanois* and that of these aphids is the position of the retrogressing spermatids. In the former case the smaller spermatids approach the sustentacular cells, and then gradually retreat toward the tails of the larger spermatids; their position, consequently, is regular, having a relation to the development of the larger spermatids. In the latter case, however, the position of the retrogressing spermatids is irregular.

As in the case of *Stomaphis yanois* retrogressed spherical cells are seen in the cyst with fully developed spermatozoa. These cells attach themselves to the epithelium of the cysts and have the same fate as the retrogressed cells of the smaller spermatids of *Stomaphis yanois*.

V. Review.

According to Meves and others, one of the secondary spermatocytes of the honey bee is much smaller than the other, and receives no chromosomes; it, consequently, degenerates after some time. The larger secondary spermatocyte, moreover, divides unequally in the second spermatocyte division. The chromosomes divide this time, and there are produced larger and smaller spermatids. The larger spermatids differentiate into functional spermatozoa. The smaller spermatids also undergo some differentiation which, however, comes to a standstill at a late stage and then they degenerate without transforming into functional spermatozoa. The smaller spermatid of *Stomaphis yanois* resembles that of the honey bee in some respects. Both of them are much smaller than the larger spermatids, but judging from the Meves' drawings, the difference in size between the larger and the smaller spermatids is greater in the honey bee than in the aphid. They both develop to some extent, but do not transform into functional spermatozoa. Meves does not state what kind of changes occurs in the degenerating smaller spermatids of the honey bee; I am, therefore, unable to compare their later stages with those of the smaller spermatids of *Stomaphis yanois*.

The most conspicuous difference between the smaller spermatids of the honey bee and this aphid is seen in the nuclei. The nucleus of the smaller spermatid of the honey bee returns to a resting stage, and differentiates similar to that of the larger spermatid. The nucleus of the smaller spermatid of this aphid, however, becomes condensed after the second spermatocyte division, and remains in the same state, although the cytoplasm shows changes similar to those of the larger spermatid. This may be caused by the absence of the lagging chromosomes in the smaller spermatids, while in the honey bee the smaller spermatids have the same number of chromosomes as the larger spermatids.

Whitney (1918) mentions that the normal and rudimentary spermatozoa have been found in considerable number of rotifers. In his paper of 1917 he says that the functional spermatozoa are identical in their power of determining the sex of the individual that develops from a fertilized egg, since after a functional spermatozoon has fertilized a parthenogenic male egg, the egg always develops into a female individual.

In the case of these rotifers, according to Whitney, the chromosomes divide in the first spermatocyte division. One half of the secondary spermatocytes divide and form the normal spermatids. The remaining half of the secondary spermatocytes, contrary to the case of the smaller secondary spermatocyte of *Stomaphis yanois*, do not divide, but develop directly into the degenerate spermatozoa. The spermatocytes destined to degenerate are smaller than the others, and their development into the complete rudimentary spermatozoa is strikingly different from the development of the normal spermatids.

Whitney ('18) says that as all the fertilized eggs in both phylloxerans and rotifers develop into female young, it seems safe to conclude, as Morgan has already concluded, that the degenerate sperm cells are the male-determining ones and that the normal sperm cells are the female-determining ones.

Stevens (1905) found many degenerate spermatozoa in *Blattella germanica*. She states that the distribution and varying number of these degenerate spermatozoa make it impossible to interpret their condition as due to the absence of the accessory chromosome as Miss Wallace does in the spider, and that the only

probable explanation seems to lie in the imperfect mitosis. She detected no evidence of degeneracy among the young spermatids.

VI. Summary.

1. In *Stomaphis yanois* the smaller secondary spermatocytes divide, and develop to some extent, but retrogress to spherical cells.

2. In *Neothomasia populicola* and *Macrosiphum ambrosia*, cases of division of the smaller secondary spermatocytes were found, but no developing smaller spermatids were observed.

3. In *Neothomasia populicola* and *Macrosiphum ambrosia* spherical cells like those in *Stomaphis yanois* were found in the cysts containing spermatozoa. These were identified as retrogressed larger spermatids.

LITERATURE.

von Baehr, W. B.

'08 Ueber die Bildung der Sexualzellen bei Aphididæ. Zoöl. Anz., Bd. 33.

'09 Die Oogenese bei einigen viviparen Aphididen und die Spermatogenese von *Aphis saliceti*, mit besonderer Berucksichtigung der chromatinverhältnisse. Arch. f. Zellforsch., Bd. 3.

'12 Contribution a l'etude de la caryocinese somatique de la pseudoreduction. La Cellule, tom 27.

Meves, F.

'07 Die Spermatocytenteilungen bei der Honigbiene. Arch. f. mikr., Anat. Bd. 70.

Morgan, T. H.

'08 The Production of Two Kinds of Spermatozoa in Phylloxerans—functional "Female Producing" and Rudimentary Spermatozoa. Proc. Soc. Exp. Biol. and Med., V.

'09 A Biological and Cytological Study of Sex Determination in Phylloxerans and Aphids. Jour. Exp. Zoöl., Vol. 7.

'10 The Chromosomes of the Parthenogenetic and Sexual Eggs of Phylloxerans and Aphids. Proc. Soc. Exp. Biol. and Med., Vol. 7.

'15 The Determination of Sex in Phylloxerans and Aphids. Jour. Exp. Zoöl., Vol. 19.

Stevens, N. M.

'05a Study of Germ Cells of *Aphis rosæ* and *Aphis œnotheræ*. Jour. Exp. Zoöl., Vol. 2.

'05b Studies in Spermatogenesis with Special Reference to the "Accessory Chromosome." Carnegie Inst. Wash., Pub. No. 36.

'06a Studies of the Germ Cells of Aphids. Carnegie Inst. Wash., Pub. No. 51.

'09 An Unpaired Heterochromosome in the Aphids. Jour. Exp. Zoöl., Vol. 6.

Tannreuther, G. W.

'07 History of the Germ Cells and Early Embryology of Certain Aphids. Zoöl. Jahrb. Anat. Ontog., Bd. 24.

Wallace, L. B.

'05 The Spermatogenesis of the Spider. BIOL. BULL., Vol. 6, No. 3.

Whitney, D. D.

'17 The Production of Functional and Rudimentary Spermatozoa in Rotifers. BIOL. BULL., Vol. 33.

'18 Further Studies on the Production of Functional and Rudimentary Spermatozoa in Rotifers. BIOL. BULL., Vol. 34.

Wilson, H. V.

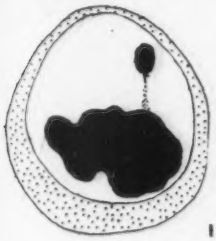
'11 On the Behavior of Dissociated Cells in the Hydroids, *Alcyonaria* and *Asterias*. Jour. Exp. Zoöl., Vol. 11.

EXPLANATION OF PLATES.

All of the drawings were made with the aid of camera lucida. Figs. 1 to 15 were drawn with a Leitz 1/16 oil immersion objective and a Zeiss compensating ocular 18. Figs. 16 to 37, except Fig. 33, were drawn with a Leitz 1/16 oil immersion objective and a Leitz ocular 5. Fig. 33 was drawn with a Leitz 1/16 oil immersion and a Leitz ocular 4. Fig. 38 was drawn with a Leitz 1/16 oil immersion objective and a Leitz ocular 3. All figures from *Stomaphis yanois*.

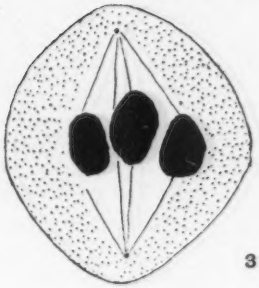
PLATE I.

FIGS. 1 AND 2. Primary spermatocytes, prophase.
FIGS. 3, 4, 5 AND 6. Primary spermatocytes, metaphase.
FIG. 7. Primary spermatocyte, anaphase.
FIGS. 8 AND 9. Primary spermatocytes, telophase.

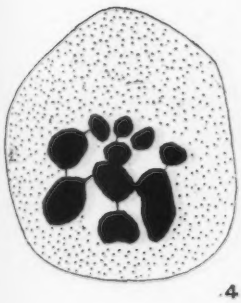


1

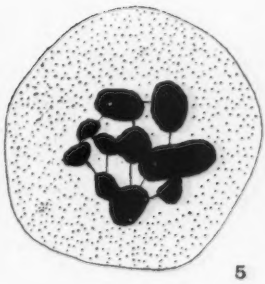
2



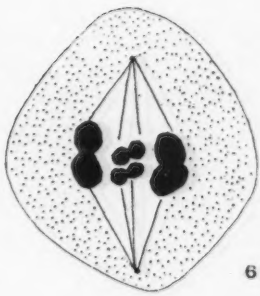
3



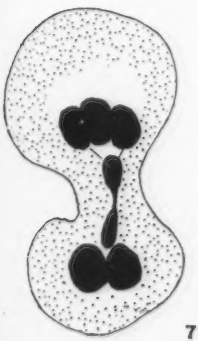
4



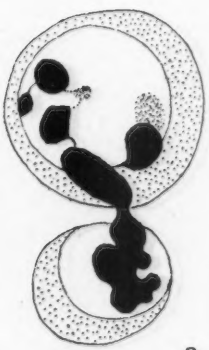
5



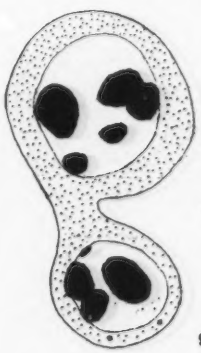
6



7



8

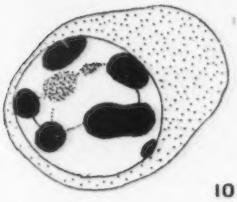


9

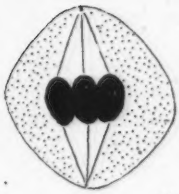
H. HUNDA.

PLATE II.

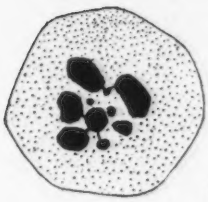
FIG. 10. Larger secondary spermatocytes, prophase.
FIGS. 11, 12 AND 13. Larger secondary spermatocyte, metaphase.
FIGS. 14 AND 15. Larger secondary spermatocytes, anaphase.
FIG. 16. Larger secondary spermatocyte, telophase.
FIGS. 17, 18 AND 19. Larger spermatids.



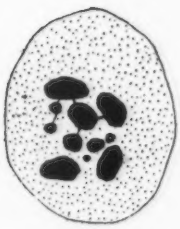
10



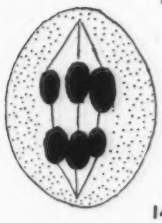
11



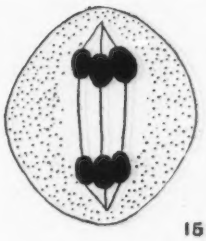
12



13



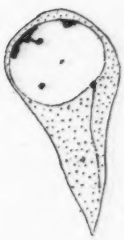
14



15

16

17



18

19

PLATE III.

FIGS. 20, 21, 22, 23 AND 24. Smaller secondary spermatocytes, metaphase.
FIGS. 25 AND 26. Smaller secondary spermatocytes, anaphase.
FIG. 27. Smaller secondary spermatocyte, telophase.
FIGS. 28, 29, 30 AND 31. Smaller spermatids.

20

21

22

23

24

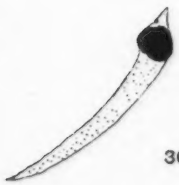
25

26

27

28

29



30

31

H. HONDA.

PLATE IV.

FIGS. 32 AND 33. Smaller spermatids.
FIGS. 34, 35 AND 36. Retrogressing smaller spermatids.
FIG. 37. Retrogressed cell of the smaller spermatid.
FIG. 38. Layers of the retrogressed cells.

32

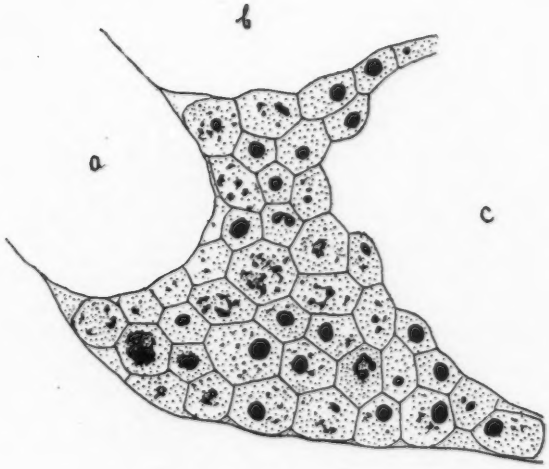
33

34

35

36

37



38